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RD_X in Plant Tissue

Leading to Humification in Surface Soils

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Abstract

The overall objective was to improve the understanding of RDX transformation in plant tissues and the subsequent cycling of tissue-associated RDX and RDX daughter products among soil mineral and humic fractions following plant senescence. The hypothesis was that environmental risks from RDX at military training ranges can be reduced, and possibly eliminated, through a series of coupled processes involving plant uptake, plant enzyme mediated transformation, photodegradation in the plant, and finally humification of plant-tissue-associated RDX conjugates into soil organic matter after plant senescence and leaf drop. Although the effect of each individual process may be small, the combined effects of the processes taken as a system for sustainability may have a significant impact on RDX residues on surface soils. If so, they may lead to feasible range sustainability management practices.

RDX is found in the soils and groundwater of bombing ranges and manufacturing sites. Plants of the family Lamiaceae were used to determine if either their enzymatic activities could accelerate the degradation of RDX once taken up from an aqueous solution. Plant tissue with higher chlorophyll content was found to contain higher concentrations of RDX, while the presence of anthocyanin appeared to have no impact. Of the four varieties of mint tested, chocolate mint, a variety of spearmint [*Mentha spicata*], had significantly lower RDX concentrations in its leaf tissues. Further research is needed to determine what processes are responsible for the reduced RDX content.

Ascorbate, pH, and glutathione (GSH) were found to be statistically significant factors in the photodegradation of 2,4,6-trinitrotoluene (TNT), a process applicable to RDX. Ascorbate and pH increased the rate of TNT degradation, whereas GSH inhibited it. Photo-induced degradation of TNT occurs at approximately the same rate in extract-based solution. The results indicate that ascorbate and pH increase the rate of photolysis of TNT, whereas glutathione decreases it.

In sufficiently reduced systems, RDX has been shown to attenuate, but the specific reactions and characterization of the residues that are produced have not been completely determined. Recent studies have demonstrated that both bacteria and fungi can also mineralize RDX, but, again, the pathways and intermediates formed are poorly understood. Because precedence has been established for RDX transformation, and explosives have been shown to bind covalently to soil humic fractions or organic material in compost, a humification approach may have significant utility in treating surface soils on impact and training ranges.

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Table of Contents

Abstract.....	ii
List of Figures and Tables	v
Preface.....	vii
Executive Summary	viii
1 Introduction.....	1
1.1 Overall objective	1
1.2 Hypothesis	2
2 Task 1: Localization of RDX in Leaves of Lamiaceae—Impact of Pigments and Secondary Metabolic Pathways	4
2.1 Introduction.....	4
2.2 Background.....	5
2.3 Methods and materials	6
2.3.1 Plant selection and preparation	6
2.3.2 Experimental design	7
2.3.3 Plant take-down and tissue preparation.....	7
2.3.4 RDX extraction procedures and analysis.....	8
2.3.5 Pigment quantification.....	9
2.3.6 Data processing and calculations.....	9
2.4 Results and discussion	9
2.4.1 Distribution of RDX within plant tissues	9
2.4.2 RDX distribution in <i>Coleus</i> leaves	10
2.4.3 Effect of chlorophyll on RDX distribution	11
2.4.4 Effect of anthocyanins on RDX distribution.....	13
2.4.5 Further evidence of the effect of pigments on RDX degradation in <i>coleus</i>	15
2.4.6 Effect of chlorophyll on RDX degradation in <i>Mentha</i> species	18
2.4.7 Effect of anthocyanins on RDX concentration in all leaf tissues.....	20
2.5 Conclusion	21
3 Task 2: Identification of Significant Factors in the Phytophotolysis of 2,4,6-Trinitrotoluene.....	23
3.1 Introduction: photodegradation	23
3.2 Experimental.....	24
3.2.1 Materials.....	24
3.2.2 Solar simulation	24
3.2.3 Experimental design	25
3.2.4 Experimental procedure	27
3.2.5 Analytical methods.....	28
3.3 Results and discussion	28

3.3.1	<i>Multivariate photolysis study</i>	28
3.3.2	<i>Extract photolysis study</i>	32
3.4	Fate of RDX in selected plants.....	33
3.5	Photolysis modeling as a probe for biodegradation	34
3.5.1	<i>Uptake and accumulation</i>	35
3.5.2	<i>Photodegradation studies</i>	36
4	Task 3: Humification	38
4.1	RDX transformation	40
4.2	Approach	42
4.2.1	<i>Materials and methods</i>	43
4.2.2	<i>Results and accomplishments</i>	44
4.2.3	<i>Leachate toxicity</i>	48
4.3	Conclusions.....	49
5	Conclusions.....	50
5.1	Task 1.....	50
5.2	Task 2.....	51
5.3	Task 3.....	51
	References.....	52
	Appendix A: Supporting Data	60
	Appendix B: List of Technical Publications.....	68
	Report Documentation Page	

List of Figures and Tables

Figures

Figure 1. Conceptual fate of RDX carbon in soil–plant–sunlight–microbial system.	2
Figure 2. Volume of stock RDX-water solution taken up by each plant species.....	10
Figure 3. Pigmentation patterns of all coleus used	11
Figure 4. Location of RDX in leaf tissue sections of all-green coleus.....	11
Figure 5. Location of RDX by mass in each corresponding section of tri-color coleus.....	12
Figure 6. Location of RDX in leaf tissues of green-white coleus.....	13
Figure 7. RDX concentration in all plant tissues for two varieties.....	14
Figure 8. RDX distribution within the dark red-green coleus	15
Figure 9. RDX distribution in the green-red coleus	16
Figure 10. RDX distribution in the leaf tissue of green-red coleus.....	17
Figure 11. RDX and chlorophyll concentrations in all coleus green leaf tissues.	17
Figure 12. RDX and chlorophyll concentrations in mint plants.....	18
Figure 13. RDX and chlorophyll concentrations in all coleus and <i>Mentha</i> plants	19
Figure 14. Distribution of RDX within tissues of <i>Mentha</i> plants.....	20
Figure 15. RDX-exposed spearmint plants vs. control plants prior to takedown	20
Figure 16. RDX concentration and anthocyanin absorbance in all plants used.....	21
Figure 17. Direct photolysis of TNT occurs rapidly ($t_{1/2} = 1.46$ hours) in water. $T = 26 \pm 1^\circ\text{C}$; $n = 3$; $\text{pH} = 7.25$; $[\text{TNT}]_{\text{initial}} = 101.8 \pm 3.3 \mu\text{M}$	29
Figure 18. Three-dimensional plot of k_{obs} as a function of ascorbate and glutathione in water at $\text{pH} = 7.25$ was derived from the model.	31
Figure 19. Presence of ascorbate in fresh extract increased the rate of TNT photolysis by 4.3-fold.....	33
Figure 20. RDX photolysis does not correlate with the presence of several different cytosol components.	36
Figure 21. Photodegradation of nitrobenzene correlates strongly with the presence of glutathione	37

Figure 22. RDX and the reductive transformation products, MNX, DNX and TNX.	40
Figure 23. Approach to track and identify fate of ¹⁴ C-RDX added either directly to soil or from plant tissue.....	44
Figure 24. Soil organic matter fractionation scheme.	44
Figure 25. Color changes in the organic fractions following soil extraction.	45
Figure 26. Humic/fulvic ratios in high and low OM soils treated with either ¹⁴ C-labelled RDX applied directly to the soil or ¹⁴ C-labelled RDX that was incorporated into plant tissue by growing plants in ¹⁴ C-labelled RDX in soil (+ Leaf).	45
Figure 27. Microbial biomass in high-organic soil and low-organic soil, with and without RDX additions. Soil respiration expressed as evolved CO ₂ , in high-organic soil and low-organic soil, with and without RDX additions, and with plant tissue and associated RDX amendment.....	46
Figure 28. Summary of significant pathways in RDX fate in soil–plant–microbial systems.....	47
Figure 29. Treatments that were tested using a modified leachate toxicity test based on the growth inhibition of the algae <i>Selenastrum capricornutum</i>	49

Tables

Table 1. Experimental conditions for the multivariate photolysis experiments in which the effects of pH, glutathione, and ascorbate on TNT photodegradation were examined.....	25
Table 2. Five concentration levels matching the coded factor levels for the three-factor central composite design.....	27
Table 3. Experimental conditions and corresponding k_{obs} values for TNT photodegradation.	30
Table 4. β -values and corresponding t -test comparisons for the full factor model at the 95% confidence level.	30
Table 5. Percentage of each factor's contribution to the overall photolysis rate.	32
Table 6. Degradation rates of TNT from the extract photolysis experiments.....	33
Table 7. RDX uptake and accumulation in three plants.	35
Table 8. Differences in RDX update in tricolored leaves of coleus.....	35

Preface

This study was conducted for the Strategic Environmental Research and Development Program.

The work for the tasks 1 and 2 were conducted by Dr. Lee Newman, Carrie Haden, Dr. John Ferry, and Trey Rahn at the University of South Carolina (USC). The comparisons of plant uptake and removal rates (task 1) were done in the Dr. Newman's laboratory and greenhouses. The comparison of RDX accumulation and degradation rates (task 2) were a joint project between Dr. Newman and Dr. Ferry, with the bulk of the analysis being done in the Chemistry Department at USC. The soil humification work (task 3) was conducted by Dr. Charles M. Reynolds' team (Biogeochemical Sciences Branch, Dr. Terrence M. Sobecki, Chief) at the US Army Engineer Research and Development Center, Cold Regions Research and Engineering Laboratory (ERDC/CRREL) in the laboratory under controlled conditions. At the time of publication, Dr. Justin Berman was Chief of the Research and Engineering Division. The Deputy Director of ERDC-CRREL was Dr. Lance Hansen and the Director was Dr. Robert Davis.

This work was supported by SERDP project number ER-1412, *Fate of Plant Tissue Associated RDX in Surface Soil*. The majority of the SERDP funding was provided to Dr. Newman and Dr. Ferry at the University of South Carolina through BAA Contract no. W913E5-04-C-0004, and supported two Master of Science Theses: T. W. Rahn, *Identification of significant factors in the photophotolysis of 2,4,6-trinitrotoluene*, and C.B.B. Hadden, *The phyto- and photodegradation of RDX by plants in the Lamiaceae family*, from which tasks 1 and 2 of this report are derived. Task 3 was co-funded by USACE-ERDC EQI project *RDX Attenuation on ranges via the management of natural soil cycles*. The authors thank Mark Hardenberg for his efforts in report preparation.

COL Kevin J. Wilson was the Commander of ERDC, and Dr. Jeffery P. Holland was the Director.

Executive Summary

Our overall objective was to improve the understanding of RDX transformation in plant tissues and the subsequent cycling of tissue-associated RDX and RDX daughter products among soil mineral and humic fractions following plant senescence.

Our hypothesis was that environmental risks from RDX at military training ranges can be reduced, and possibly eliminated, through a series of coupled processes involving plant uptake, plant enzyme mediated transformation, photodegradation in the plant, and finally humification of plant-tissue-associated RDX conjugates into soil organic matter after plant senescence and leaf drop. Although the effect of each individual process may be small, the combined effects of the processes taken as a system for sustainability may have a significant impact on RDX residues on surface soils. If so, they may lead to feasible range sustainability management practices.

Many methods for the removal of RDX from soil or groundwater have been employed over its long history of use. Traditional methods, such as bulk composting or incineration, have many drawbacks. Both require the initial step of excavation, which not only affects the immediate ecosystem, but can be hazardous to the workers performing the excavation if the contaminated soil originated from a bombing range. Incineration is a fairly quick process but requires excavation and transport, and can spread the contamination further via air-borne particles. It also results in large quantities of sterile soil that must be disposed of by landfilling. Composting requires exacting physical conditions to produce effective degradation of contaminants. Both processes are labor intensive, require specialized equipment, and are therefore expensive. Groundwater contamination by RDX can be remediated via pump and treat methods such as oxidative degradation by UV, peroxide (H_2O_2), or ozone. However, these processes are also costly and often results in incomplete degradation of the parent compound.

For uptake and within leaf fate of RDX, we investigated plants in the family Labiatea (genera *Mentha* and *Coleus*) to determine their relative sensitivities to RDX and to determine the uptake rates for RDX for the selected

plants. RDX location within the leaf tissue varied with leaf pigmentation. Plants in the family Labiatea have been shown to have high enzyme activities associated with their pigmentation.

We also examined the phytophotolysis of TNT using simulated plant cell conditions as the degradation of TNT pollution has application to RDX. The study was made up of two different experimental approaches, including multifactor experiments in aqueous solution and single factor experiments using iceberg lettuce extract. Through multivariate analysis of variance (MANOVA), we derived a predictive model for the effect of pH (4.5–8.5), ascorbate (0–50 mM), and glutathione (0–10 mM) on the photodegradation of TNT in water. The extract photolysis experiment was used to simulate the photolysis that can occur in the actual cell environment. This approach tested the effects of ascorbate and glutathione on the phytophotolysis of TNT.

Because RDX uptake by plants has been demonstrated, but the subsequent fate of RDX is less well defined, we sought to improve the understanding of RDX transformation in plant tissues and the subsequent cycling of tissue-associated RDX and RDX daughter products among soil mineral and humic fractions following plant senescence. The concept is to exploit plant uptake of RDX and within-plant processes, such as conjugation of toxins in plant tissue as a first step in an overall humification strategy. For our humification studies, we obtained plant tissue containing ^{14}C labeled RDX. We then used those plants with high uptake rates to follow RDX humification and fate in soils held at moisture and temperature conditions representative of surface soils on training ranges.

1 Introduction

1.1 Overall objective

Our overall objective was to improve the understanding of RDX transformation in plant tissues and the subsequent cycling of tissue-associated RDX and RDX daughter products among soil mineral and humic fractions following plant senescence. To accomplish this, we looked at plants within the family Labiatea (genera *Mentha* and *Coleus*) to determine their relative sensitivities to RDX and to determine the uptake rates for RDX for the selected plants. We then used those plants with high uptake rates to follow RDX humification and fate in soils held at moisture and temperature conditions representative of surface soils on training ranges.

There has been considerable interest in the use of plants to clean up munitions wastes, both in surface waters and soils. Work by other groups (Thompson et al. 1999; Schneider 1995; Harvey et al. 1991) has shown that plants are capable of taking up RDX. To determine which species were capable of uptake, Schneider (1995) looked at plants that were colonizing a field site that had been contaminated with RDX. Plants that showed an accumulation of RDX within their tissues included black locust (*Robinia pseudoacacia*), red cedar (*Juniperus virginiana*), Canadian goldenrod (*Solidago canadensis*), ragweed (*Ambrosia artemisiifolia*), and many others. Harvey et al. (1991) looked at the potential uptake of RDX into the crop plant bush bean (*Phaseolus vulgaris*, var. tendergreen) to determine if there was a potential for RDX to move into the food chain. Thompson et al. (1999) looked at the uptake of RDX by poplar trees (*Populus deltoids* x *P. nigra*, clonal line DN34) with the intent of finding a means to phytoremediate RDX-contaminated sites. Both Harvey et al. (1991) and Thompson et al. (1999) found that RDX was readily taken up by the plants, although at a slower rate than water. Thompson et al. (1999) found that, in water-based systems, the concentration of RDX can increase as the water is preferentially taken up. In soil pore water, this could also lead to an increase in available RDX. Harvey et al. (1999) also found no evidence of transpiration of RDX by the plants. Both Thompson et al. (1999) and Harvey et al. (1991) also looked for the evidence of metabolites in plants, but found that the vast majority of RDX accumulated in the leaf tissue of the plants as parent compound. However, Harvey et al. (1991) noticed an increase in non-extractable radiolabel in the remaining plant pellet, indicat-

ing that, over time, the beans may have been incorporating the RDX into the plant matrix. None of the studies reported a toxicity component.

1.2 Hypothesis

Our hypothesis was that environmental risks from RDX at military training ranges can be reduced, and possibly eliminated, through a series of coupled processes involving plant uptake, plant enzyme mediated transformation, photodegradation in the plant, and finally humification of plant-tissue-associated RDX conjugates into soil organic matter after plant senescence and leaf drop. Although the effect of each individual process may be small, the combined effects of the processes taken as a system for sustainability may have a significant impact on RDX residues in surface soils. If so, they may lead to feasible range sustainability management practices. Mechanistically, pigments and aromatics in plant tissue may control or alter plant-related transformations and photodegradation. Bioavailable carbon from decaying plant tissue may be critical in driving soil humification processes. For these reasons, we proposed to study the processes as a system. A diagram of the processes is shown in Figure 1.

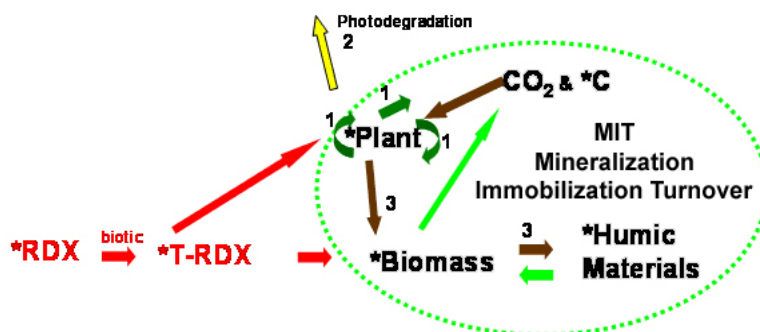


Figure 1. Conceptual fate of RDX carbon in soil-plant-sunlight-microbial system. We addressed three processes: 1) plant synthesis and catabolism, 2) photodegradation, and 3) humification of plant-tissue-conjugated RDX carbon in conjunction with Mineralization-Immobilization Turnover (MIT).

Much is known about RDX fate in soils and its uptake by plants. The initial microbially driven degradation steps for RDX in soil at defined conditions, shown in red on Figure 1, have been characterized by others. Although degradation in the plant does not appear to provide a major transformation pathway in plants studied, it does occur. Much RDX is conjugated into compounds having larger molecular weights by plants in a process described as a “green liver” function and believed to be part of plant detoxification strategies. The pathways determining RDX fate in plant tissue are

less defined. This project focused on understanding the occurrence and magnitude of processes denoted by arrows 1, 2, and 3 in Figure 1. To address the knowledge gaps for these processes, our hypothesis included the concepts that:

- Process 1. RDX fate in plant tissue is subject to unique plant enzyme systems that may be associated with synthesis of plant pigments or aromatic compounds.
- Process 2. Photodegradation in plant tissue may vary with pigment levels in the tissue, and this phenomenon may be a useful tool for understanding occurrence and magnitude of photodegradation of RDX in plant tissue.
- Process 3. Humification of plant-tissue-associated RDX conjugates is a component of and is influenced by natural carbon cycles. Carbon cycles in soil include mineralization, or loss of carbon from the system as CO₂, and immobilization, or fixing carbon into the soil organic fraction. These processes occur simultaneously—a net process termed Mineralization-Immobilization Turnover (MIT)—and reach a dynamic equilibrium. The equilibrium can be shifted towards mineralization by processes such as tilling, or towards immobilization by processes such as carbon additions. On ranges, we may be able to alter humification processes by adding high levels of carbon, such as from plant senescence.

To evaluate the three parts of this hypothesis, we had three main objectives or tasks. The bulk of this report is divided into sections based on these three objectives.

1. Plant uptake and removal.
2. Fate of RDX in selected plants: accumulation, phyto- and photodegradation rates.
3. Humification: fate of ¹⁴C-RDX added either directly to soil or from plant tissue.

2 Task 1: Localization of RDX in Leaves of Lamiaceae—Impact of Pigments and Secondary Metabolic Pathways

2.1 Introduction

Because of its extensive use as a high explosive, hexahydro-1,3,5-trinitro-1,3,5-triazine (commonly known as RDX) is an environmental contaminant found primarily in the soil and groundwater near munitions manufacturing facilities and bombing practice ranges. RDX is known to affect the central nervous system, is classified as a possible human carcinogen (ATSDR 1995), and is on the USEPA's Drinking Water Contaminant Candidate List (USEPA 2005).

Many methods for the removal of RDX from soil or groundwater have been employed over its long history of use. Traditional methods (e.g., bulk composting or incineration) have many drawbacks. Both require the initial step of excavation, which not only devastates the immediate ecosystem, but is very hazardous to the workers doing the excavating if the contaminated soil originated from a bombing range. Incineration is a fairly quick process but it can actually spread the contamination further via air-borne particles and it also results in large quantities of sterile soil that must be disposed of by landfilling. Composting requires exacting physical conditions to produce effective degradation of contaminants (Tuomi et al. 1997). Both processes are very labor intensive, require specialized equipment, and are therefore very expensive (Cunningham et al. 1995; Doyle and Kitchens 1993; Emery and Faessler 1997). Groundwater contamination of RDX can be remediated via pump and treat methods such as oxidative degradation by UV, peroxide (H_2O_2), or ozone (Bose et al. 1998a, b). However, this process is also very costly and often results in incomplete degradation of the parent compound (Talley and Sleeper 1997).

Phytoremediation, the use of plants to take up and degrade a contaminant, is an alternative to the costly traditional methods of remediation. Many different species of plants can remove RDX both from soil or aqueous solutions and some species have demonstrated the ability to degrade it (Just and Schnoor 2004; Harvey et al. 1991; Larson et al. 1999a, b).

For this study we have selected two genera of the Lamiaceae family: *Mentha*, the common mints, and coleus, which are often used for landscaping. Mint plants are not only very hardy and propagate easily, but native varieties are found growing in abundance all over the world. Both genera have metabolic processes that may affect the degradation of RDX within the living leaf tissue. Scent compounds in mint plants are secondary metabolites and contain a central ring structure similar to the ring structure of RDX. Coleus plants have anthocyanin and chlorophyll pigments, which are also secondary metabolites with central ring structures. The naturally occurring metabolic processes that are responsible for the maintenance and degradation of internal secondary metabolites may also degrade RDX. In addition to the potential enzymatic degradation of RDX, the leaf tissue of some coleus plants also contains large unpigmented areas. Aqueous RDX has been shown to photodegrade under laboratory conditions (Hawari et al. 2002; Choi et al. 1995; Bose et al. 1998a, b); therefore, the potential exists for RDX to photolyse while in the unpigmented leaf tissue.

Hydroponically grown coleus and mint plants were exposed to aqueous RDX and grown under artificial lights. After several days the plants were separated by tissue type (leaf, stem, and root) and the leaf tissue dissected by pigmentation pattern, if appropriate. The RDX concentration in the solutions and in all tissues was then determined. We compared an all green coleus to several varieties of mints to determine if the secondary metabolites in the mints affect RDX degradation rates. The all green coleus was then compared to the other, multi-colored coleus plants to determine the effects of the pigments on RDX degradation.

2.2 Background

There are increasing concerns that US military readiness will be reduced if training ranges are closed or their use is restricted. Low-order, incomplete detonation of explosives on ranges has resulted in the surface distribution of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) that is both heterogeneous and widely dispersed. The leaching of RDX from contaminated surface soils on ranges has been implicated as a possible mechanism for groundwater contamination and is an immediate concern at Army and DoD ranges. Remediation strategies that are cost effective, easily implemented, and applicable to range surface soils are needed.

Two low-cost approaches, natural attenuation and phytoremediation, are increasingly viewed as acceptable treatment technologies by regulators and stakeholders. A critical concern in the use of these technologies is the permanence of the remediation. There is a need for unambiguous evidence demonstrating that these techniques transform RDX into benign complexes that no longer pose a future environmental risk. Dissolved RDX has been shown to undergo a series of reductive transformations under sufficiently reduced conditions, yet surface soils are generally well aerated and become wet only ephemerally. As such, surface soils on ranges can serve as an RDX source from which surface and subsurface waters can become contaminated. A potential treatment technology would be one that ties together the primary benefits of phytoremediation with humification. Such a technology would combine the benefits of plant-driven RDX cycling with RDX transformation and microbially driven humification.

Earlier research has shown that many explosive transformation products bind covalently to soil humic fractions or organic material in compost, thereby minimizing the associated health risks. Previous phytoremediation studies have shown that RDX can be taken up by plants, but that the compound accumulates in the plant rather than being degraded. Plant uptake and any subsequent phytoremediation or photoremediation may partially reduce or retain RDX, but some RDX likely will return to the soil as the plant senesces. Humification of plant associated RDX may have significant utility for continued treatment of tissue-associated RDX on military training ranges.

2.3 Methods and materials

2.3.1 Plant selection and preparation

All plants used in this study were propagated and grown hydroponically from cuttings of plants purchased locally or grown from seeds in a greenhouse. Coleus (*Solenostemon scutellaroides*) varieties grown from seed were from Wizard Mix (a mix of varieties from Park Seed), Rainbow Mix (a varietal mix from Burpee Seeds), and the variety “Limelight” (Park Seeds). The mint species used were spearmint (*Mentha spicata*), lemon balm (*Melissa officinalis*), chocolate mint (a variety of spearmint), and curly leaf mint (a variety of spearmint).

Cuttings were placed in glass Erlenmeyer flasks covered with aluminum foil to prevent algal growth and were grown in half-strength Hoagland’s

solution (Phyto Technology Labs #H353-50L). Solutions were changed weekly and buds removed as necessary to prevent flowering. Plants were grown under florescent lights until roots were well developed, then plants were grown either under grow-light (16 hours on—8 hours off) or natural sunlight.

2.3.2 Experimental design

Each species and variety of plant was dosed separately; treated plants and controls were run simultaneously. Batches of plants were exposed in multiples of 3–5 (control plants) and 4–5 (treated plants). Individual plants were placed in a clean, aluminum foil covered Erlenmeyer flasks. They were given a measured amount of water, and pieces of foil were placed over the opening around the stems to prevent evaporation or photo-degradation of the solutions. Control plants received DI water (Nanopure Infinity, 18.0 mΩ) and treated plants received vacuum-filtered (to 0.45 μm) RDX/DI water. The stock RDX/DI water was maintained as a saturated solution in a single large container. For each species of plant dosed, approximately 3.5 L of the stock solution was vacuum filtered to remove any undissolved RDX and placed in a single aluminum foil covered, 4-L amber bottle to ensure all treated plants received exactly the same concentration of RDX. At the time of dosing, an aliquot of the filtered RDX/DI water was taken for analysis and stored in an amber 40-mL vial; this vial was then placed in a paper bag and stored at 4°C.

Plants were placed under a laboratory fume hood with an installed grow-light (GE Lucalox HPS 400-W bulb on a 16 hour—8 hour on/off cycle) to ensure stable conditions during the exposure period. This light was chosen because it does not block emission in the UV range of the spectrum. Plants were regularly monitored for stress. Plants remained under experimental conditions until they took-up a minimum of 200 mL of solution.

2.3.3 Plant take-down and tissue preparation

All plants were taken down sequentially. The volume of the remaining solution in each flask was measured using a graduated cylinder. An aliquot of the solution was immediately placed in an amber 40-mL vial and stored in at 4°C until analyzed. The plant roots were rinsed thoroughly under running tap water to remove any RDX residue and then gently blotted dry. Plants were separated into tissue types: leaf, stem, and root. Because the plants started from cuttings, all tissue below the waterline was considered

root tissue. Leaves of multi-colored coleus plants were sectioned by pigmentation using scissors; leaves of the mints and those of the all-red coleus remained intact. Leaves of the all-green coleus were divided by size. Leaves over 5-cm long were cut into three even sections: vein, middle, and edge. Leaves under 5 cm, including all buds, were placed into a separate grouping. Once a whole plant was dissected, all tissues were weighed, the tissue was placed into glass jars, and stored in a -40 or -80°C deep freezer.

Prior to extraction, all frozen tissue was ground into small pieces and homogenized. Stems and roots were ground using a chilled coffee bean grinder (Proctor Silex #E160B) and leaf tissue was ground with a mortar and pestle and liquid nitrogen. The prepared tissue was returned to the -40 or -80°C freezer until ready for extraction.

2.3.4 RDX extraction procedures and analysis

Methods used to extract the RDX from the solutions and frozen tissues were based on Method 8330 (USEPA 1994) and the *Modifications to Method 8330* by Larson et al. (1998).

Weighed tissue samples were placed in vials with HPLC grade acetonitrile. Vials were placed in a cooled ultrasonic bath (VWR model no. 250D) for 18 hours then centrifuged (Thermo IEC model no. CL2) at 2000 rpm for 10 minutes and allowed to sit for 1 hour. The supernatant was filtered through a clean-up column (wetted with fresh acetonitrile) containing LC-Alumina-A (Supelco no. 57206) and Florisil (Supelco no. 2-0281). The same quantity of acetonitrile was passed through the same column and mixed with the supernatant. The sample was diluted 1:1 with DI water then filtered into an amber HPLC vial using a disposable Luer-Lok syringe and 0.45- μm PTFE filter.

The RDX/DI water samples and aqueous samples from the control plants were prepared for analysis by diluting 1:1 with acetonitrile, vortexing for 1 minute, and filtering into an amber HPLC vial in the same method as the tissue extracts.

RDX was analyzed using a Perkin-Elmer high-performance liquid chromatography (HPLC) system with a PE Series 200 auto sampler and the UV detector set at 245 nm. The column used was a Supelcosil LC-18 column (Supelco no. 58298) with diameter of 4.6 mm and length of 25 cm. Meth-

anol/water (50/50) was used for the mobile phase with a flow rate of 1.0 mL/min and an injection volume of 50 μ L. Calibration standards (1–125 ppm in 50:50 Acetonitrile:H₂O) were made by serial dilution using RDX in acetonitrile (1000 ppm) standard (Restek Corp. no. 31666).

2.3.5 Pigment quantification

Anthocyanins were extracted from the homogenized, frozen leaf tissue using methods modified from Mancinelli et al. (1991). Approximately 2 g (± 0.01 g) of frozen leaf tissue was placed in a 15-mL centrifuge tube and 4 mL of acidic methanol (1% HCl in HPLC grade methanol) was added. The sample was shaken for 2 hours at room temperature and centrifuged for 3 minutes at 2000 rpm. The absorbances of the resulting extract were determined using a NanoDrop spectrophotometer (ND 1000) and MS Excel software. Chlorophylls were extracted using the same method with the exception that the solvent used was pure methanol.

2.3.6 Data processing and calculations

Anthocyanin absorbance (A_A) was calculated using the equation: $A_A = A_{530} - 0.25A_{657}$ (Mancinelli et al. 1991). Total chlorophyll concentration ($Chl_a + Chl_b$) was determined using the equation: $C_{a+b} = 1.44A_{665} + 24.93A_{652}$ (Lichtenthaler 1987).

For both the RDX and the pigment quantification data, Total Chrome software was used to interpret the results. Data were statistically validated by a Grubbs test to verify outliers, a *t*-test or analysis of variance (ANOVA) to compare data sets, and all data were analyzed at a 95% confidence interval.

2.4 Results and discussion

2.4.1 Distribution of RDX within plant tissues

The concentration of RDX in all aqueous samples was verified using HPLC. No significant change in concentration from start to finish of each experiment was detected. This verified that no plant in this study selected or excluded RDX preferentially from the RDX–water. Because of the variation in plant species and their individual rates of absorption (Fig. 2), the RDX concentration in the leaf tissue was standardized by dividing the calculated RDX concentration in that leaf section by the volume of RDX–water solution taken up by each plant. This allowed us to compare the con-

centration of RDX in tissues between the species and varieties with different uptake rates.

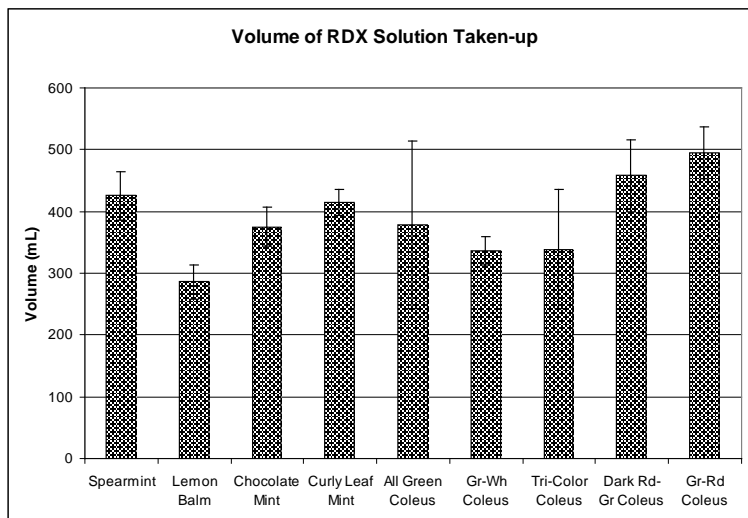


Figure 2. Volume of stock RDX-water solution taken up by each plant species.

2.4.2 RDX distribution in Coleus leaves

The RDX concentration in all green-leaved coleus (Fig. 3a) was determined for the vein, middle, and edge sections of the leaves individually (Fig. 3f). The biomass of each section was calculated and used to determine what portion of the leaves were in each category (Fig. 4). If RDX was distributed equally across the leaf surface, then the percent mass of the RDX in each section would be equal to the percent of biomass in that section. However, the actual relative mass of RDX in the leaf edges was found to be significantly higher ($p=0.009$) over the expected value, resulting in $10.2 \pm 4.8\%$ more RDX than expected. While there was no significant difference in the middle 34% of the leaf, the central, vein portion had $8.5 \pm 3.0\%$ less RDX than expected ($p = 0.003$). This indicates that RDX is translocated preferentially to the margins of the leaves of coleus plants.

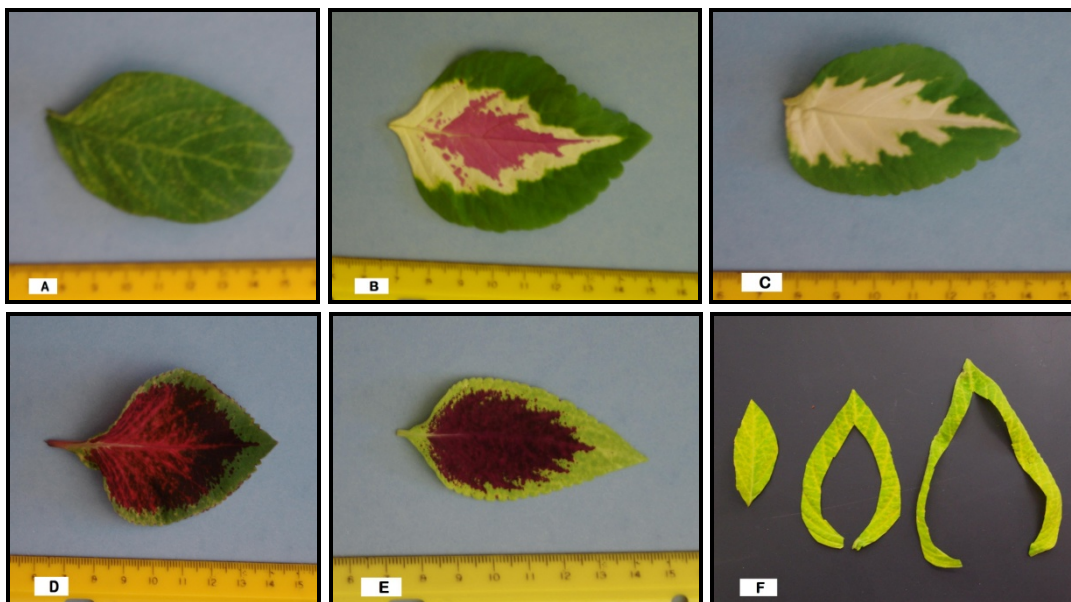


Figure 3. Pigmentation patterns of all coleus used: a) all-green; b) tri-color; c) green-white; d) dark red-green; e) green-red; f) dissection pattern of all-green coleus leaves.

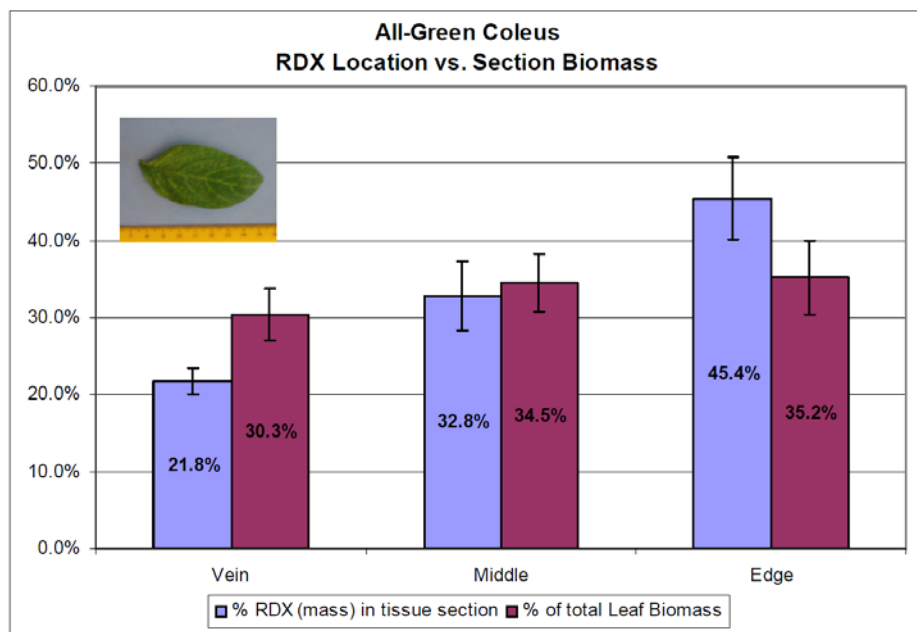


Figure 4. Location of RDX in leaf tissue sections of all-green coleus. RDX concentrated disproportionately in margins of leaves.

2.4.3 Effect of chlorophyll on RDX distribution

A tri-colored coleus variety was also divided into three sections by pigmentation (Fig. 3b) and the biomass and RDX concentration of each section were determined (Fig. 4). While the outer, green portion of the leaves was

$58.2 \pm 3.1\%$ of the biomass of the leaf tissue, it contained $79.4 \pm 1.8\%$ of the RDX by mass.

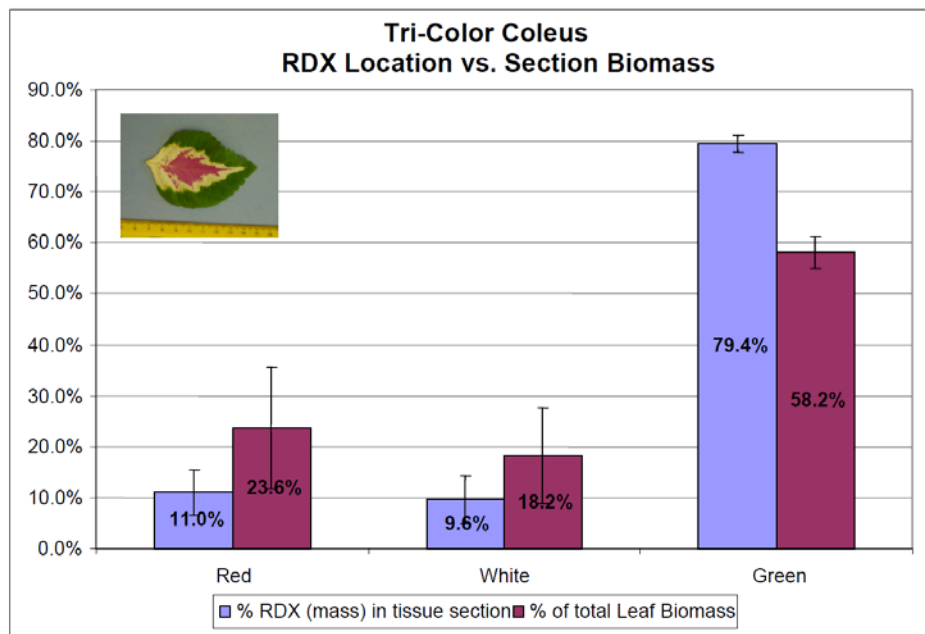


Figure 5. Location of RDX by mass in each corresponding section of tri-color coleus. The RDX is located primarily in the green leaf margins. However, no significant difference exists in RDX concentration between the red and white leaf sections ($n = 4$).

This significant increase ($p = 0.0006$) in the amount of RDX in the outer portion of the leaves compared to the amount that was expected reflects the decrease of RDX in the inner two sections. If measured RDX concentrations were dependant solely on spatial distribution, then the two middle sections would have increasing RDX quantities as it was translocated from the central vein towards the margins and, therefore, the white portion of the leaf would have a higher proportion of RDX than the red section. The middle/white section ($18.2 \pm 9.4\%$ of the biomass) contained $9.6 \pm 4.7\%$ of the RDX, while the inner/red section ($23.6 \pm 11.9\%$ of the biomass) contained $11.0 \pm 4.5\%$ of the RDX. This decrease in the actual RDX mass (red = $12.6 \pm 7.5\%$, white = $8.6 \pm 4.8\%$) was found to be significant ($p = 0.0429$, $p = 0.0378$, respectively) for both sections. The concentrations of RDX in the three sections were determined to be 67 (red), 73 (white), and 188 ppm (green). While there is a significant difference in concentration of RDX between the green tissue and both the white ($p = 0.0026$) and the red ($p = 0.0024$), there is no significant difference between the red and the white sections ($p = 0.4133$). This reduced concentration and overall mass

of RDX in the white section compared to the red section may be attributable to degradation of RDX taking place in the white sections.

2.4.4 Effect of anthocyanins on RDX distribution

To determine if RDX degradation was being affected by the presence of the anthocyanins in the tri-color coleus leaf tissue, a green-white coleus (Fig. 3c) was also exposed to RDX and analyzed by color section (Fig. 6). As expected, the green, outer section ($60.2 \pm 3.5\%$ of the biomass) contained $81.2 \pm 1.6\%$ of the RDX by mass, which is a significant increase ($p = 0.0027$) of $21.0 \pm 4.5\%$ more RDX than expected if the RDX mass were proportional to the biomass of that section. This is comparable to the $21.2 \pm 2.7\%$ increase in the outer, green section of the tri-color coleus.

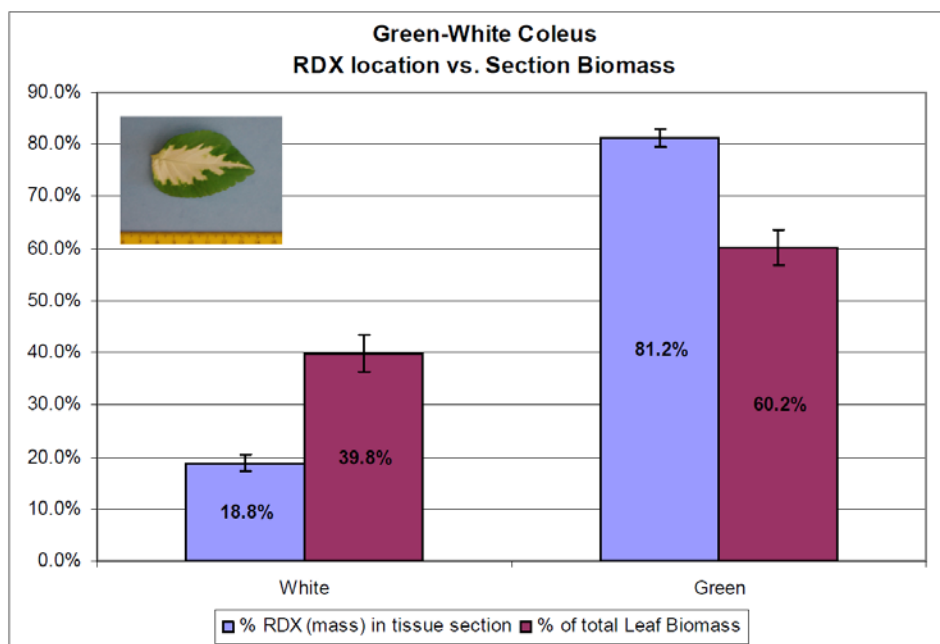


Figure 6. Location of RDX in leaf tissues of green-white coleus. The RDX is located primarily in the green, leaf margins ($n = 4$).

Figure 7 shows the standardized concentration of RDX in all leaf tissues of the tri-color and the green-white coleus. While the two varieties took up the same amount of RDX–water (Fig. 2, $p = 0.2686$) and contained similar concentrations in the root tissues ($p = 0.8916$), the green-white coleus had significantly lower concentrations of RDX in all remaining tissues (Stem:Stem $p = 0.0208$; Gr:Gr $p = 0.0103$, Wh:Wh $p = 0.0075$, Wh:Rd $p = 0.0284$). The lower concentrations of RDX in the coleus with the increased

mass of white tissue may be ascribable to increased photodegradation in these non-pigmented areas.

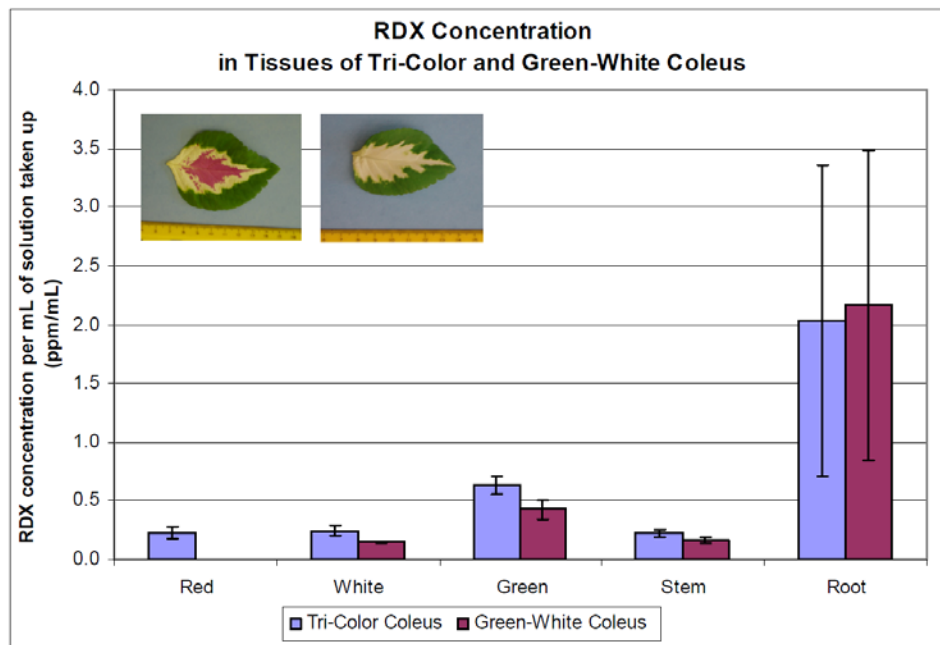


Figure 7. RDX concentration in all plant tissues for two varieties. Concentration of RDX in each tissue standardized by mL of RDX-water solution taken-up by each plant ($n = 4$, tri-color coleus; $n = 4$, green-white coleus).

This still does not explain why the red and white sections of the tri-color coleus had similar concentrations of RDX. If RDX was translocated preferentially toward the margins of the leaf, then the middle, white section would have a higher concentration of RDX than the central, red portion. If photodegradation is taking place in the unpigmented sections, then we would expect the concentration in the white areas of the tri-color coleus to be lower than the red sections, unless the primary pigments in the red tissue, the anthocyanins, do not block the UV degradation of RDX within the leaf tissue.

Another coleus variety was treated with the RDX–water solution—a dark red-green variety (Fig. 3d). It was found to contain $44.6 \pm 3.8\%$ of the RDX by mass in the green, outer 24.1% of the leaf tissue (Fig. 8). Even though the interior, dark red portion of the leaves contained nearly three times the amount of anthocyanins ($p = 0.0005$), the outer section contained significantly higher concentrations of both RDX ($p = 0.0046$) and chlorophyll ($p = 0.0019$) than the red section. We believe that this higher concentration in RDX is the result of a combination of the translocation of

RDX across the leaf surface, demonstrated by the all green coleus (Fig. 3a), and the significantly higher concentration of chlorophyll in the tissue. The presence of the anthocyanins appears to have no effect on the RDX concentration.

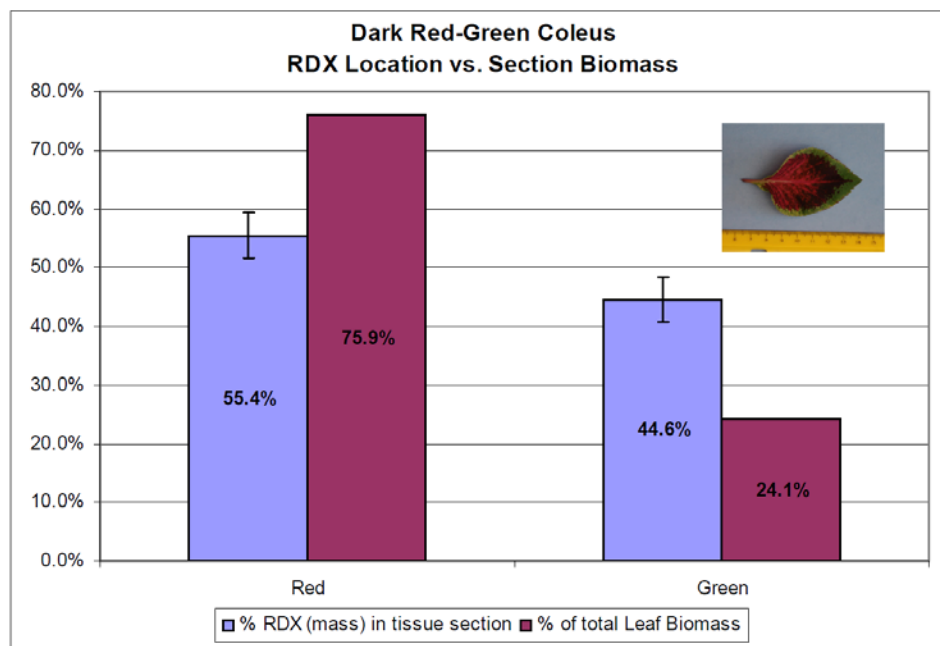


Figure 8. RDX distribution within the dark red-green coleus. The RDX is located primarily in the marginal green leaf tissue ($n = 5$, RDX mass; $n = 1$, leaf biomass).

2.4.5 Further evidence of the effect of pigments on RDX degradation in coleus

A different variety of green and red pigmented coleus was also treated with RDX: a green-red coleus (Fig. 3e) with the outer, green edge consisting of approximately 42% of the leaf biomass and the inner, red section being the remaining 58%. The total leaf and root tissues were found to contain, on average, the expected amount of RDX in them (Fig. 9); the stem tissue did have $10.2 \pm 4.4\%$ lower amounts of RDX than expected ($p = 0.0276$). The mass of RDX was found to be proportional to the biomass of each pigmented section; the outer 42% of the leaf tissue contained $38.3 \pm 7.6\%$ of the RDX by mass and the red, inner section contained $61.7 \pm 7.6\%$ of the RDX by mass (Fig. 10). Additionally, there was no significant difference in either the RDX concentration ($p = 0.2361$) or the chlorophyll concentration ($p = 0.1009$) of the two leaf sections. Therefore, even though the two sections were visibly different, and the red tissues did contain a significantly higher amount of anthocyanins ($p = 0.0119$), there was no distin-

guishing pattern of RDX distribution across the leaf surface. This would seem to indicate that, like in both the tri-color and the dark red-green coleus, the anthocyanins appear to have no effect on the RDX degradation. Unlike the other varieties of coleus tested, this variety did not display the normal translocation of RDX to the outer portions of the leaf. This lack of RDX in the outer section of the leaf tissue may be explained by the low chlorophyll concentration in that tissue. The outer, green portion of the leaves contained significantly less chlorophyll than any other green coleus tissue tested ($p = 0.0015$, tri-color coleus; $p = 0.0018$, gr-wh coleus; $p = 0.0031$, all-green coleus, all sections; $p = 0.0005$, dark rd-gr coleus) as well as significantly less RDX ($p = 0.0002$, tri-color coleus; $p = 0.0008$, gr-wh coleus; $p = 0.0180$, $p = 0.0080$, $p = 0.0045$, all green coleus vein, middle, edge, respectively; $p = 0.0008$, dark rd-gr coleus) than the other green tissues (Fig. 11). Therefore, because the RDX in the outer sections of the green-red coleus had less chlorophyll, the RDX was photodegraded faster than in the green tissues of other coleus varieties.

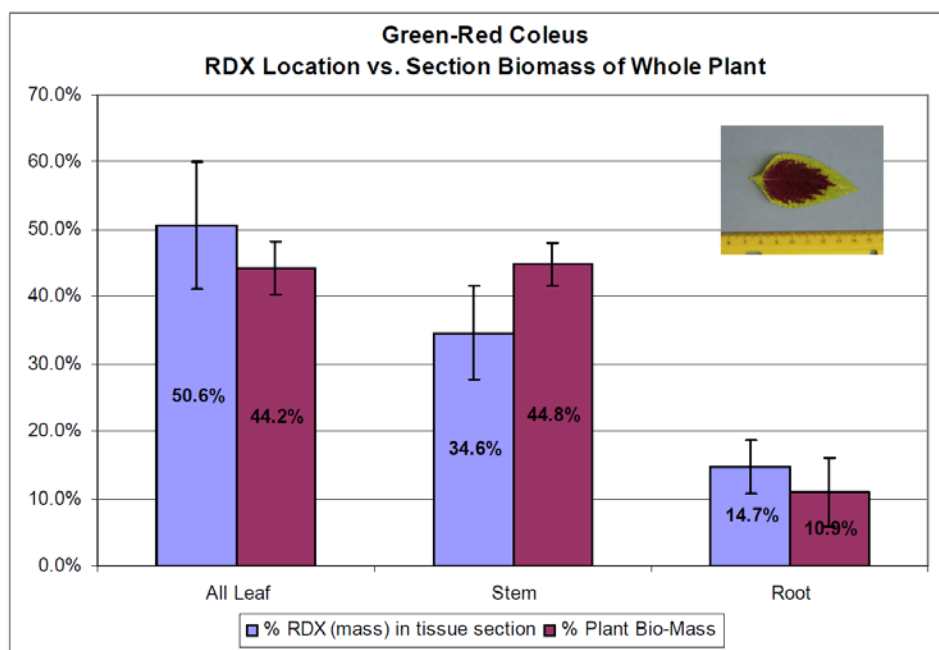


Figure 9. RDX distribution in the green-red coleus. The RDX is distributed proportionately throughout the plant ($n = 4$).

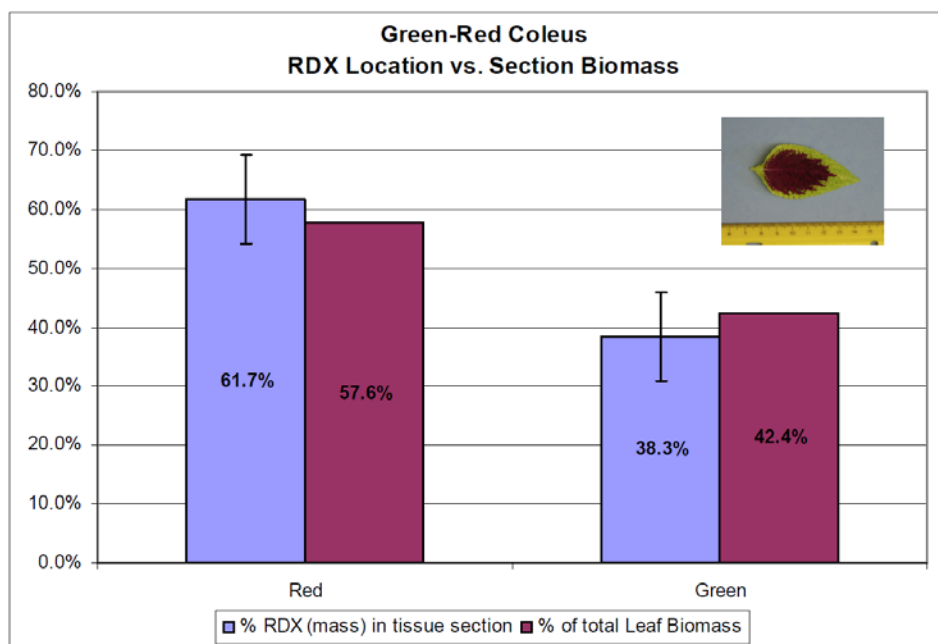


Figure 10. RDX distribution in the leaf tissue of green-red coleus. The RDX is distributed proportionately to the tissue mass and does not correspond to pigmentation pattern ($n = 4$, RDX mass; $n = 1$, leaf biomass).

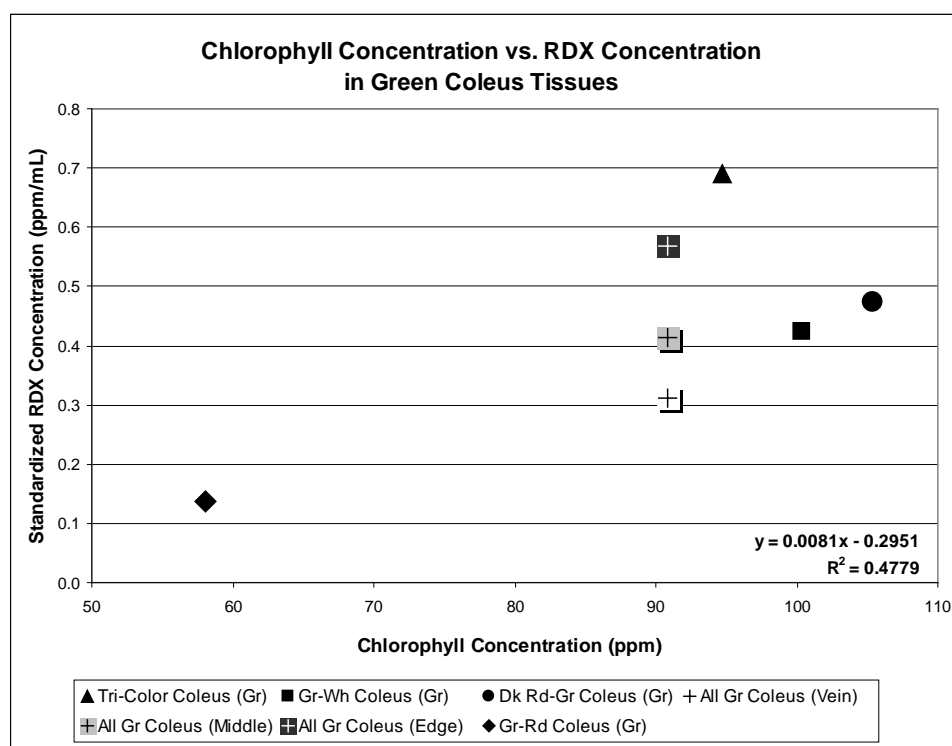


Figure 11. RDX and chlorophyll concentrations in all coleus green leaf tissues. There does appear to be a positive correlation between RDX concentration and chlorophyll concentration.

2.4.6 Effect of chlorophyll on RDX degradation in *Mentha* species

Four varieties of mint plants were exposed to RDX–water solution, divided by tissue type, and the biomass, RDX concentration, and mass were determined for each tissue. By plotting the concentration of chlorophyll versus the standardized RDX concentration in the leaf tissue of all four mint varieties (Fig. 12), a linear relationship between the two variables is clear ($R^2 = 0.8056$). When the chlorophyll concentration and the standardized RDX concentration for all coleus and all *Mentha* varieties are plotted together (Fig. 13), the relationship is still clear ($R^2 = 0.6056$). However, the chocolate mint has significantly less RDX in its leaf tissues than in the green leaf tissue of all the coleus varieties except the green-red variety ($p = 0.6939$). This variety is also the only green coleus tissue which whose chlorophyll concentration is significantly less than the chlorophyll concentration in the leaf tissue of the chocolate mint ($p = 0.0013$); the others statistically contain the same concentration. Therefore, the question remains, why is the chocolate mint so substantially different from the other mints?

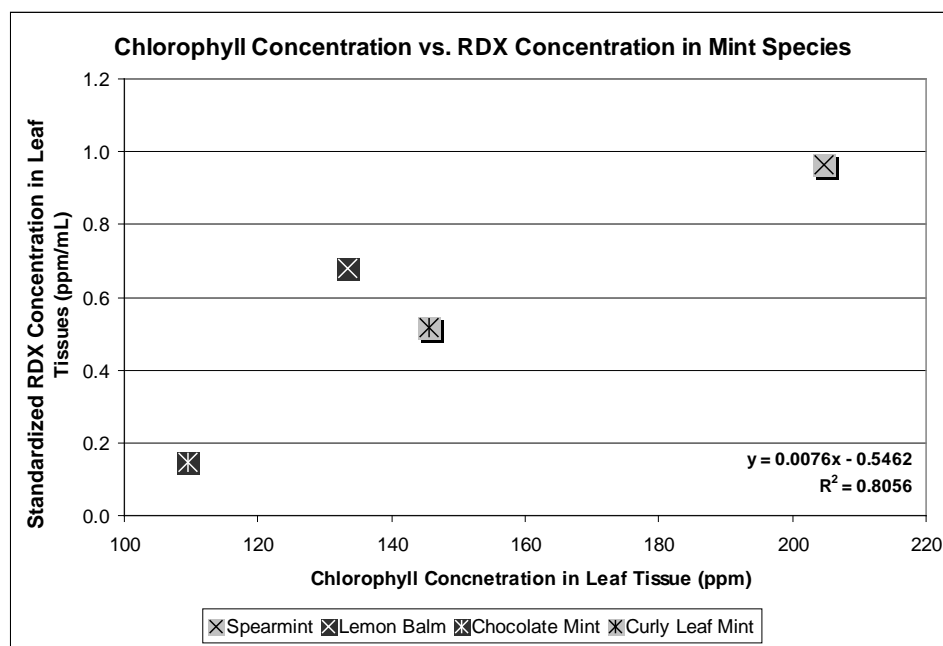


Figure 12. RDX and chlorophyll concentrations in mint plants. There is a positive relationship between RDX concentration and chlorophyll concentration.

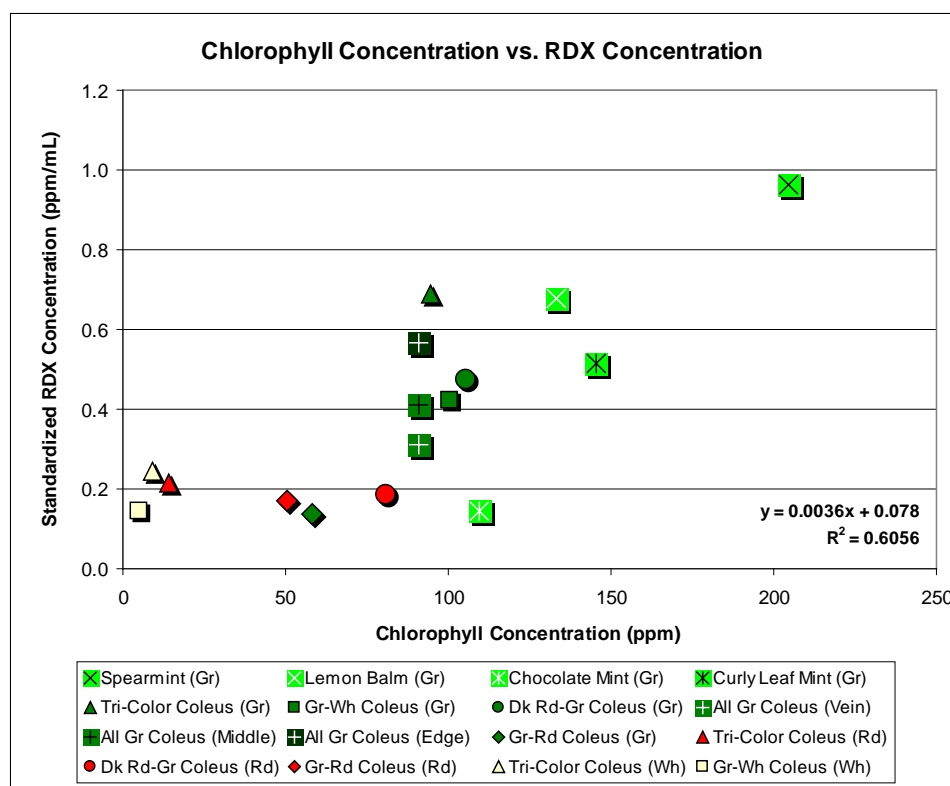


Figure 13. RDX and chlorophyll concentrations in all coleus and *Mentha* plants. There is an overall positive relationship between chlorophyll concentration and RDX concentration.

When we compare the standardized RDX concentration in all plant tissues of the four mints (Fig. 14), the chocolate mint has a significantly lower concentration of RDX in all tissues, except that there is no significant difference between the chocolate mint root tissues and the root tissues of the spearmint ($p = 0.1201$) (see Fig. 15 for RDX-exposed spearmint plants vs. control plants). However, while the chocolate mint has lower concentrations in the stem and root tissues in general, it is the magnitude of the difference between the chocolate mint leaf tissue and that of the leaf tissue of the other varieties ($p < 0.0001$ for all) that is remarkable. If we were to look only at the mint plants (Fig. 12), this difference may only be attributable to the lower chlorophyll concentration. However, as the chlorophyll concentration in the chocolate mint is statistically the same as the chlorophyll concentration in all green coleus tissues (Fig. 13) while having significantly lower RDX concentrations—except for the green-red, as previously noted—then there is no clear explanation for the reduced RDX concentration, except to speculate that an additional mechanism is at work beyond chlorophyll concentration.

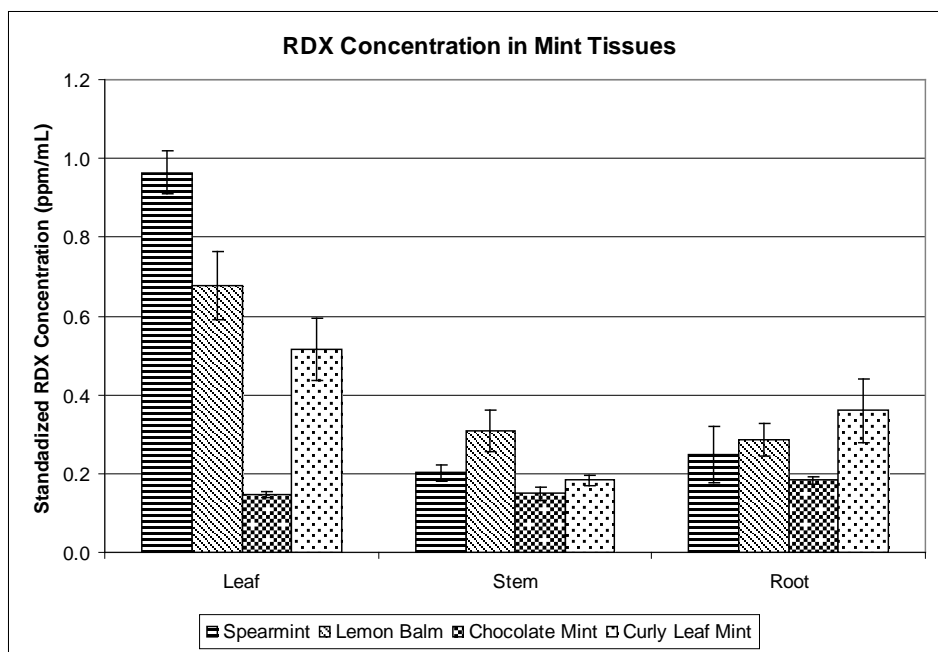


Figure 14. Distribution of RDX within tissues of *Mentha* plants.

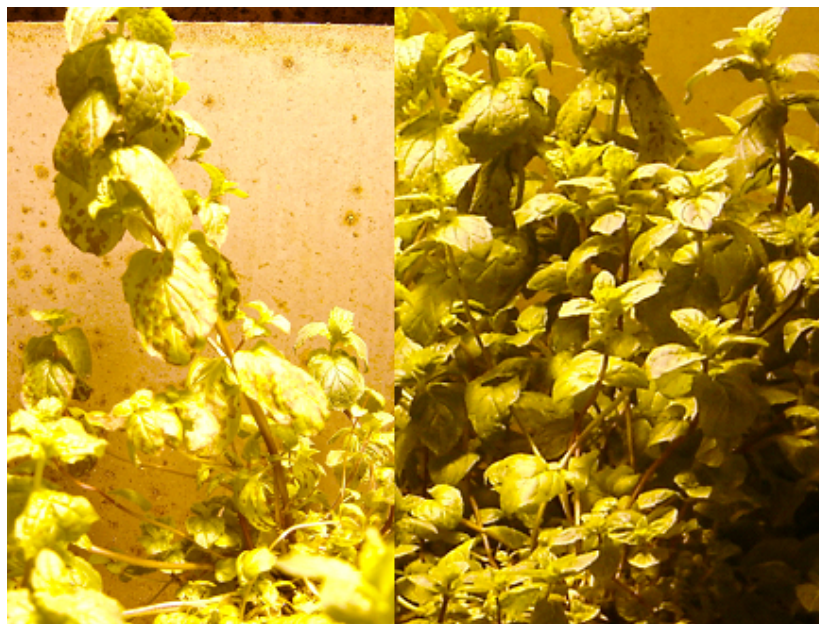


Figure 15. RDX-exposed spearmint plants vs. control plants prior to takedown. Note brown lesions on exposed plants.

2.4.7 Effect of anthocyanins on RDX concentration in all leaf tissues

Because absorbance is directly related to concentration in tissue samples, the anthocyanin absorbance can be graphically represented against the RDX concentration using the same method as the chlorophyll concentration (Fig. 16). When this is done, the results do not support any visible

trend. This confirms the previously discussed findings that there is no direct relationship between anthocyanin absorbance and RDX concentration.

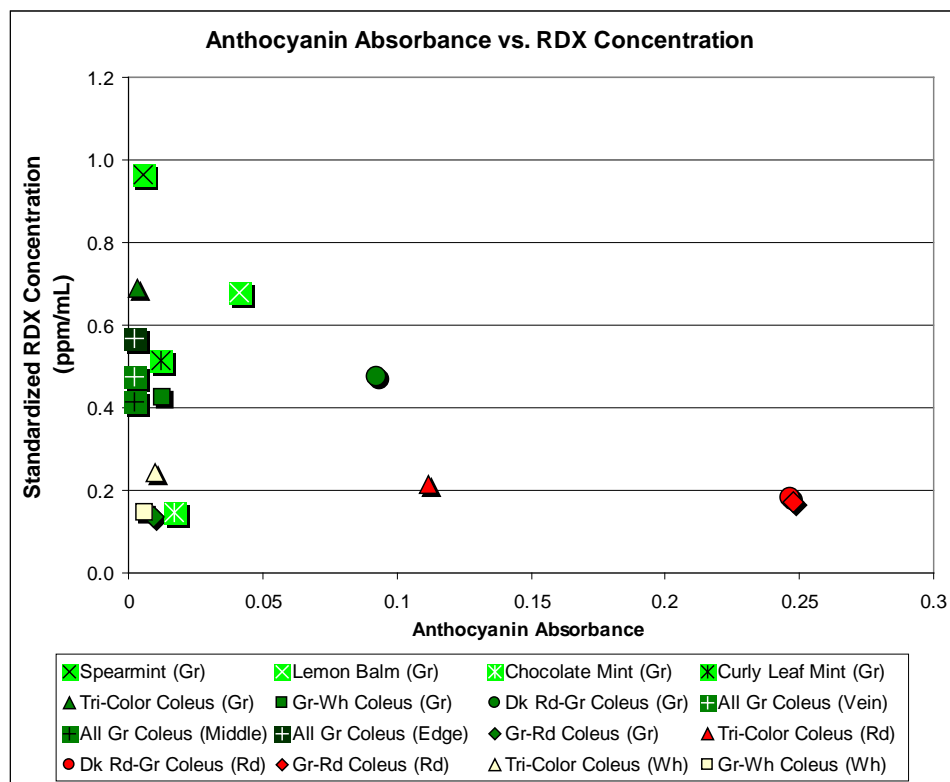


Figure 16. RDX concentration and anthocyanin absorbance in all plants used. There is no relationship between RDX concentration and anthocyanin absorbance.

2.5 Conclusion

As demonstrated by the all-green coleus leaf tissue, RDX is translocated preferentially across the leaf surface and accumulates at higher levels in the outer edges. It would be beneficial in future research to test this theory by exposing different uni-colored coleus varieties to RDX and verifying that they have the same dispersion pattern. It would also be beneficial to test this theory with plants from other genera.

Chlorophyll concentration affects RDX concentration; higher chlorophyll content results from higher RDX concentrations in the leaves. As evidenced by the green-red coleus, this effect of chlorophyll concentration on RDX degradation is more important than simple translocation; when chlorophyll concentration is exceptionally low, then RDX degrades quickly and does not accumulate.

The presence of anthocyanins in the leaf tissue has no effect on RDX concentration. When anthocyanins are present in tissues that would otherwise be similar—green-white coleus compared to tri-color coleus and in the dark red-green coleus compared to all green coleus—there is no change in the ratio of RDX concentration.

When the chocolate mint was compared to the other mint species tested, it exemplified the idea that higher chlorophyll concentration results in higher RDX concentration within the leaf tissue. However, when compared to the green coleus tissues tested, it seems that the opposite is true and we can, therefore, only conclude that an accelerated rate of degradation is taking place in the chocolate mint. It would be very beneficial to screen more mint varieties to see what others may show this difference in RDX levels. We speculate that there may be RDX degradation attributable to secondary enzymatic activities within the leaf tissues of the chocolate mint; elucidating these enzymatic pathways and identifying RDX metabolites would be necessary to confirm this theory.

Based on the findings of this research, when selecting plants to use for the phytoremediation of RDX, a good selective marker would be the concentration of chlorophyll in the leaf tissues. In particular, plants with large unpigmented areas or with relatively low chlorophyll concentrations would be excellent plants to select for field studies or use.

3 Task 2: Identification of Significant Factors in the Phytophotolysis of 2,4,6-Trinitrotoluene

3.1 Introduction: photodegradation

RDX is known to photodegrade rapidly in solution on exposure to solar ultraviolet radiation (Bose et al. 1998a,b; Hawari et al. 2002). This reaction proceeds through loss of HNO_2 to produce a dinitrated enamine, which is susceptible to nucleophilic attack by H_2O to yield a series of polar, ring-opened products. Intriguingly, most of these products are a result of subsequent hydrolysis reactions of the enamine parent, implying that a single photon can lead to extensive degradation of the molecule (Bose et al. 1998a, b; Hawari et al. 2002). However, the leaf interior is extremely heterogeneous (with regard to hydrophilic and hydrophobic domains) and we would expect the “in-leaf” hydrolysis of RDX to be slowed relative to solution because of equilibration into lipid-rich environments in the leaf. The implication is that even in low-pigment leaves, RDX photodegradation and hydrolysis may be significantly slowed relative to solution, providing rich opportunities for the detection of degradation products of toxicological significance.

Phytoremediation studies have examined and confirmed the transformation of 2,4,6-trinitrotoluene (TNT) in various plant species (Hughes et al. 1997; Thompson et al. 1998; Adamia et al. 2006; Subramanian et al. 2006). TNT is also known to undergo direct and indirect photolysis rapidly in aqueous solution (Mabey et al. 1983; Spanggard et al. 1980; Burlinson 1980; Talmage et al. 1999). Because plant tissues and cells are composed of mostly water, these same photodegradation mechanisms also occur in plants. The term “phytophotolysis” has been used to describe this plant-mediated, photo-induced transformation of a xenobiotic compound (Just et al. 2004). Phytophotolysis was confirmed to be a significant mechanism in the phytoremediation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and is likely significant in TNT phytodegradation as well (Just et al. 2004).

The purpose of this study was to examine the phytophotolysis of TNT using simulated plant cell conditions. The study was made up of two differ-

ent experimental approaches, including multifactor experiments in aqueous solution and single factor experiments using iceberg lettuce extract. Through multivariate analysis of variance (MANOVA), we derived a predictive model for the effect of pH (4.5–8.5), ascorbate (0–50 mM), and glutathione (0–10 mM) on the photodegradation of TNT in water. The extract photolysis experiment was used to simulate the photolysis that can occur in the actual cell environment. This approach tested the effects of ascorbate and glutathione on the phytophotolysis of TNT.

3.2 Experimental

3.2.1 Materials

Barnstead E-pure water ($>18\text{ M}\Omega\text{ cm}$) was used in all aqueous samples and standards. TNT was purchased from AccuStandard (100% purity, New Haven, CT). L-Ascorbic acid sodium salt (99.0% purity) was produced by J.T. Baker. L-glutathione (reduced, 99% purity), L-glutamine (98% purity), and sucrose (99+% purity) were purchased from Sigma-Aldrich. Methyl-*tert*-butyl ether (OmniSolv, 99.97% purity, EMD) and hexane (100% purity, Mallinckrodt Chemicals) were used for liquid–liquid extraction. 1,4-Dichlorobenzene ($>99\%$ purity, Aldrich) was used as the internal standard during analysis. Potassium phosphate monobasic (99.5% purity, Fisher Scientific), potassium phosphate dibasic (reagent grade, Fisher Scientific), and sodium hydroxide (97.9% purity, Fisher Scientific) were used for buffering. Actinometer solution was prepared with sodium nitrate (A.C.S. grade, Fisher Scientific), benzoic acid (laboratory grade, Fisher Scientific), and sodium bicarbonate (100% purity, Fisher Scientific). The iceberg lettuce (*Lactuca sativa*) was produced in Salinas, CA.

3.2.2 Solar simulation

A Suntest XLS+ Solar Simulator (Atlas Material Testing Solutions) equipped with a 2200 W Xe vapor lamp (300–800 nm) was the light source for the photodegradation experiments. The light intensity ($I = 655\text{ W/m}^2$; RSD = 7.9%) was evenly distributed across the plane of the chamber floor (922.5 cm² polished, stainless steel surface) using a steel mesh diffuser positioned directly below the light source. Temperature was held constant at $26 \pm 1^\circ\text{C}$. The photoreactors were 8-mL vials with screw top Teflon[®] sealed lids, purchased from Laboratory Supply Distributors (Mt. Laurel, NJ). Two 30.6- × 13.2-cm polished, stainless steel trays were used to hold 68 photoreactors simultaneously. Vials containing nitrate

actinometer were added to all runs to ensure consistency in light intensity (Jankowski et al. 1999).

3.2.3 Experimental design

3.2.3.1 Direct photolysis

The direct photolysis of TNT in water served as the index reaction for all subsequent reactions. Because the direct photolysis occurs so rapidly ($t_{1/2}$ = 1.46 hours), we hypothesized that no cellular factors would significantly affect the rate of photolysis.

3.2.3.2 Multivariate photolysis experiments

The multivariate experiment was used to examine the effects of ascorbate (0–50 mM), glutathione (0–10), and pH (4.5–8.5) on the photo-degradation of TNT in water. The three-factor central composite design was produced using Design Expert 7 software (Version 7.0.2, Stat-Ease Inc., Minneapolis, MN; Montgomery 2001; Johnson and Wichern 2002). The matrix was composed of 48 total experiments (Table 1) covering 15 different experimental conditions, with five concentration levels for each factor, six replicates of the center point, and three replicates for all other points. The time for each experiment was 6 hours with sampling at 0, 1.5, 3, 4.5, and 6 hours.

Table 1. Experimental conditions for the multivariate photolysis experiments in which the effects of pH, glutathione, and ascorbate on TNT photodegradation were examined. (*Indicates center point experiments).

Experiment	pH	Glutathione (mM)	Ascorbate (mM)
1	5.5	2.5	12.5
2	5.5	2.5	12.5
3	5.5	2.5	12.5
4	7.5	2.5	12.5
5	7.5	2.5	12.5
6	7.5	2.5	12.5
7	5.5	7.5	12.5
8	5.5	7.5	12.5
9	5.5	7.5	12.5
10	7.5	7.5	12.5
11	7.5	7.5	12.5
12	7.5	7.5	12.5
13	5.5	2.5	37.5

Experiment	pH	Glutathione (mM)	Ascorbate (mM)
14	5.5	2.5	37.5
15	5.5	2.5	37.5
16	7.5	2.5	37.5
17	7.5	2.5	37.5
18	7.5	2.5	37.5
19	5.5	7.5	37.5
20	5.5	7.5	37.5
21	5.5	7.5	37.5
22	7.5	7.5	37.5
23	7.5	7.5	37.5
24	7.5	7.5	37.5
25	4.5	5	25
26	4.5	5	25
27	4.5	5	25
28	8.5	5	25
29	8.5	5	25
30	8.5	5	25
31	6.5	0	25
32	6.5	0	25
33	6.5	0	25
34	6.5	10	25
35	6.5	10	25
36	6.5	10	25
37	6.5	5	0
38	6.5	5	0
39	6.5	5	0
40	6.5	5	50
41	6.5	5	50
42	6.5	5	50
*43	6.5	5	25
*44	6.5	5	25
*45	6.5	5	25
*46	6.5	5	25
*47	6.5	5	25
*48	6.5	5	25

3.2.3.3 *Extract photolysis experiment*

The lettuce extract contained intact proteins, enzymes, and organelles, as well as the sugars, amino acids, and other molecular components commonly found in plant cells. Experiments were designed to examine the photodegradation of TNT in fresh and heat-inactivated, extract-based compositions. Dark experiments were also conducted to differentiate between molecular and solar-driven reactivity. The extract was buffered at 7.25 to coincide with published cytosolic pH values found in spinach leaves (Oja et al. 1999). Ascorbate and glutathione concentrations were based on those found in living cells of barley leaves (Rautenkranz et al. 1994).

3.2.4 Experimental procedure

3.2.4.1 *Direct photolysis*

Initial TNT concentration was prepared to 100 μ M in deionized water. Samples were adjusted to pH = 7.25 with 0.10 M phosphate buffer.

3.2.4.2 *Multivariate photolysis experiments*

All samples were made up at varied conditions of ascorbate, glutathione, and pH, as dictated by the parameter space explored by the experiment and the algorithm describing the central composite design (Tables 1 and 2). Sample pH was adjusted using 0.10 M phosphate buffer.

Table 2. Five concentration levels matching the coded factor levels for the three-factor central composite design ($n = 3$ for axial and factorial point experiments; $n = 6$ for center point experiments).

		Factor concentration levels ^a				
	Factor (units)	-2 ^b	-1 ^b	0 ^b	1 ^b	2 ^b
factor x ₁ :	pH	4.50	5.50	6.50	7.50	8.50
factor x ₂ :	Glutathione (mM)	0	2.50	5.00	7.50	10.00
factor x ₃ :	Ascorbate (mM)	0	12.50	25.00	37.50	50.00

^a Denotes initial concentrations. ^b Coded factor levels.

3.2.4.3 *Extract photolysis experiments*

For each experiment, the green, leafy portions (defined by optical absorbance) of *Lactuca sativa* were shredded using a food processor (Black & Decker, Model FP1445). The shredded lettuce was transferred to a blender (Waring, Model 51BL32) to be homogenized. The homogenate was filtered twice through two layers of Miracloth (Calbiochem, San Diego, CA; Binder

et al. 1978; Walker et al. 1984). This extract solution was used to prepare all subsequent dilutions. The absorption for the 1:25 dilution of each extract solution was 0.402 ± 0.012 at 430 nm. Thermal inactivation was achieved by heating the extract to 100°C for 5 minutes (Medina et al. 2004; Budge and Parrish 1999; Beelen and Burris 1995). Samples were adjusted to pH = 7.25 with 0.50 M phosphate buffer.

3.2.5 Analytical methods

3.2.5.1 Liquid–liquid extraction

Each aqueous sample was subjected to liquid–liquid extraction immediately following removal from the solar simulator. The extraction solvent consisted of either methyl-tert-butyl ether or hexane for the multivariate photolysis experiments or the extract photolysis experiments, respectively. The extraction solvent contained 10 ppm 1,4-dichlorobenzene, which served as the internal standard for gas chromatographic analysis. We extracted 3.00 mL of sample with organic solvent (1:1 extraction) by mixing for 45 seconds on a vortex mixer. Each sample was allowed to equilibrate for 15–20 minutes and then centrifuged at 2700 rpm using a Fisher Scientific accuSpin 1R. Approximately 1–2 mL of the organic layer was then transferred to GC vials for analysis.

3.2.5.2 Gas chromatography

A Hewlett-Packard 5890 Series II gas chromatograph coupled with an electron capture detector (GC-ECD) was used for analyzing TNT and its degradation products. The capillary column was a 30-m \times 0.25-mm \times 0.25- μ m DB-5MS (J&W Scientific). Helium was used as the carrier gas at 1.2 mL/min and nitrogen was used as the make-up gas at 40 mL/min. The injector port and detector temperatures were 225 and 310°C, respectively. The oven temperature program was as follows: the initial temperature was held for 2 minutes isothermally at 80°C, then heated at 25°C/min. to 290°C and held isothermally for 5 minutes.

3.3 Results and discussion

3.3.1 Multivariate photolysis study

TNT photodegraded rapidly under the conditions of this study. The direct photolysis of TNT (Fig. 17) occurs so rapidly ($t_{1/2} = 1.46$ hours) that we expected there to be no significant effects from cellular components. This re-

action (under pristine conditions in 18 MΩ deionized water) was used as the index reaction for all subsequent reactions; that is, the contrast between it and others allowed us to probe the effects of solution composition on the photodegradation rate.

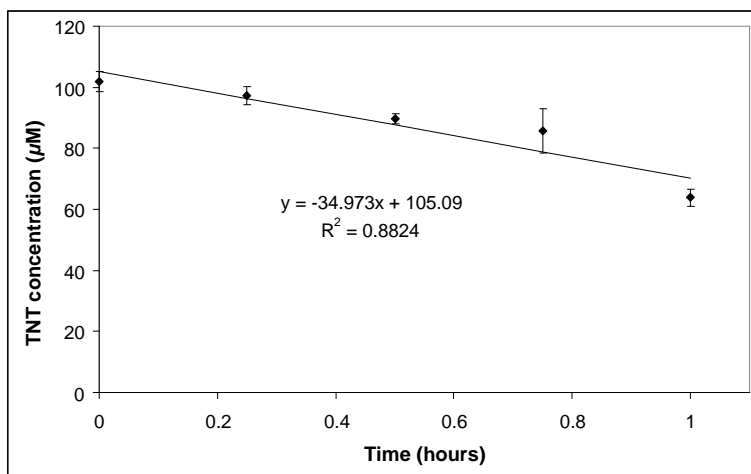


Figure 17. Direct photolysis of TNT occurs rapidly ($t_{1/2} = 1.46$ hours) in water. $T = 26 \pm 1^\circ\text{C}$; $n = 3$; $\text{pH} = 7.25$; $[\text{TNT}]_{\text{initial}} = 101.8 \pm 3.3 \mu\text{M}$.

The pseudo first-order rate constant (k_{obs}) was obtained for each condition assayed from the relationship between $\ln([\text{TNT}]_t/[\text{TNT}]_0)$ and time (Table 3). A full quadratic model was fitted to the data, including an intercept (β_0), linear terms ($\beta_1, \beta_2, \beta_3$), squared terms ($\beta_{11}, \beta_{22}, \beta_{33}$), and cross-factor terms ($\beta_{12}, \beta_{13}, \beta_{23}$) to consider all possible factor interactions. The beta values (β_x) correspond to the coefficients for each factor in the equation of the quadratic model. The coded factor levels of the three components in the quadratic model are represented as x_1 (pH), x_2 (glutathione), and x_3 (ascorbate).

$$(k_{\text{obs}}) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_{12} + \beta_{13} x_{13} + \beta_{23} x_{23} \quad (1)$$

The best fit quadratic was obtained with a \log_{10} transformation of the model. The correlation coefficient (r^2) and f -value of this model were 0.82 and 19.35, respectively. With an f -value of 19.35, there is only a 0.01% chance that this model could occur because of noise. Based on p -values, the effects from pH, glutathione, ascorbate, pH-ascorbate, and glutathione-glutathione are significant within the 95% confidence level ($p < 0.05$).

Table 3. Experimental conditions (factor levels) and corresponding k_{obs} values for TNT photodegradation.

Experiment	pH	Glutathione (mM)	Ascorbate (mM)	$k_{\text{obs}} \times 10 \text{ (hr}^{-1}\text{)}$	n
1	5.5	2.5	12.5	2.92 ± 0.87	3
2	7.5	2.5	12.5	4.36 ± 0.62	3
3	5.5	7.5	12.5	2.14 ± 0.17	3
4	7.5	7.5	12.5	2.88 ± 0.75	3
5	5.5	2.5	37.5	5.48 ± 0.55	3
6	7.5	2.5	37.5	5.09 ± 1.3	3
7	5.5	7.5	37.5	3.76 ± 0.65	3
8	7.5	7.5	37.5	4.00 ± 0.44	3
9	4.5	5	25	1.93 ± 0.17	3
10	8.5	5	25	5.21 ± 0.53	3
11	6.5	0	25	8.05 ± 1.8	3
12	6.5	10	25	3.32 ± 0.21	3
13	6.5	5	0	1.87 ± 0.23	3
14	6.5	5	50	4.90 ± 0.45	3
15	6.5	5	25	3.58 ± 0.63	6

Table 4. β -values and corresponding t -test comparisons for the full factor model at the 95% confidence level. The model has a correlation coefficient (r^2) of 0.821.

Factor components		Coefficient estimate	Standard error	$ t_{\text{calc}} $	p -value
β_0	intercept	-0.452763	0.03271		
β_1^*	x_1 -pH	0.072230	0.01236	5.8424	<0.0001
β_2^*	x_2 -glutathione	-0.082835	0.01236	6.7003	<0.0001
β_3^*	x_3 -ascorbate	0.098341	0.01236	7.9545	<0.0001
$\beta_{1,2}$	x_1x_2	0.000962	0.01748	0.0550	0.9564
$\beta_{1,3}$	x_1x_3	-0.039334	0.01748	2.2497	0.0303
$\beta_{2,3}$	x_2x_3	0.006082	0.01748	0.3478	0.7299
$\beta_{1,1}$	x_1x_1	-0.011857	0.01236	0.9591	0.3436
$\beta_{2,2}$	x_2x_2	0.040495	0.01236	3.2755	0.0023
$\beta_{3,3}$	x_3x_3	-0.017029	0.01236	1.3774	0.1764

Coefficients (β_x) for each factor (significant and insignificant) are listed in Table 4. The significant factors that increased the rate of TNT photodegradation were pH, ascorbate, and glutathione-glutathione. The significant factors that decreased the photodegradation rate were glutathione and pH-ascorbate. Only the factors that were determined to be significant

are included in the quadratic model. The derived model (eq 2) can be used to predict the effects of pH, glutathione, and ascorbate.

$$\log_{10}(k_{\text{obs}}) = -0.4528 + (0.07223)(x_1) - (0.08284)(x_2) + (0.09834)(x_3) - (0.03933)(x_1)(x_3) + (0.04050)(x_2^2) \quad (2)$$

A 3-dimensional plot of the k_{obs} values with respect to glutathione and ascorbate over the concentration range of the matrix is presented in Figure 18. The greatest rate of TNT photodegradation occurs when ascorbate levels are high and glutathione levels are low.

The linear contributions of ascorbate and pH increase the photodegradation rate. However, the increase in interaction between pH and ascorbate (pH-ascorbate factor) was found to decrease the rate. One possible explanation for this phenomenon is that ascorbic acid and ascorbate often function as reducing agents. As the pH increases, the reducing potential of ascorbic acid and ascorbate decreases. The percentage of each factor's contribution (Table 5) to the photolysis rate was calculated using sum of squares from the analysis of variance. Ascorbate has the largest contribution of all factors tested, affecting 39.3% of the overall rate of photolysis.

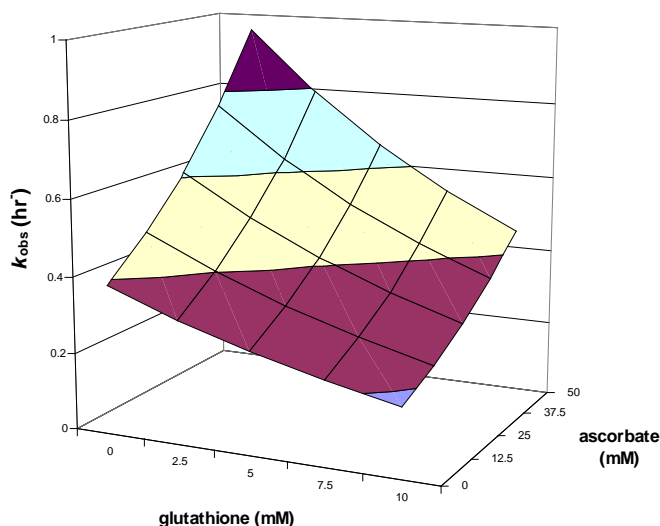


Figure 18. Three-dimensional plot of k_{obs} as a function of ascorbate (0–50 mM) and glutathione (0–10 mM) in water at pH = 7.25 was derived from the model.

Table 5. Percentage of each factor's contribution to the overall photolysis rate.

Source	Sum of squares	Outcome (%)
x_1 —pH	0.25042	21.197
x_2 —glutathione	0.32936	27.878
x_3 —ascorbate	0.46421	39.293
x_1x_2	0.00002	0.002
x_1x_3	0.03713	3.143
x_2x_3	0.00089	0.075
x_1x_1	0.00675	0.571
x_2x_2	0.07871	6.663
x_3x_3	0.01392	1.178
total	1.18142	

The linear contributions of glutathione decreased the photolysis rate as indicated by the negative coefficient for that factor in the model. However, the squared contribution of glutathione was a positive effect. This suggests that glutathione's effect becomes less negative as glutathione concentration increased across the range tested.

3.3.2 Extract photolysis study

Results of the photolysis studies were consistent with respect to the role of ascorbate in TNT photolysis. In the extract photolysis, the presence of ascorbate (25 mM) in fresh extract increased the rate of photolysis by 4.3-fold compared to fresh extract with no ascorbate (Fig. 19). Ascorbate increased the rate of photolysis by 3.0-fold in fresh extract compared to heat-inactivated extract (Table 6). As that there was negligible loss of TNT in the dark control experiments, these results suggest that the presence of ascorbate and sunlight activates an enzyme that degrades TNT.

The photolysis rates were 0.489 and 0.557 hr^{-1} for the extract experiments with and without glutathione, respectively. Based on rate comparisons, glutathione's effect was negative; however, the magnitude of the effect is negligible considering the calculated error (see Table 4).

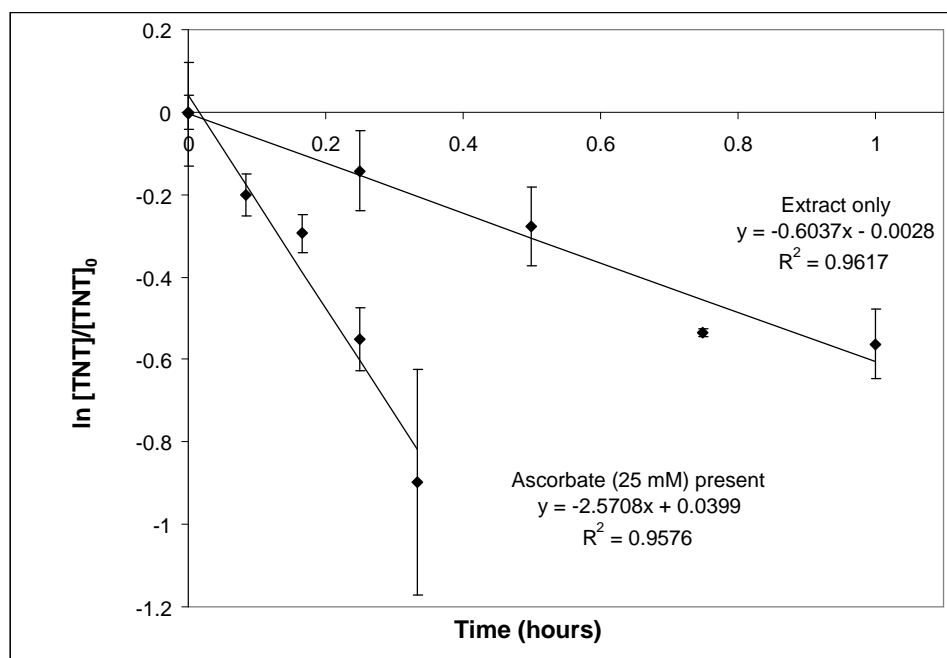


Figure 19. Presence of ascorbate (25 mM) in fresh extract increased the rate of TNT photolysis by 4.3-fold.

Table 6. Degradation rates of TNT from the extract photolysis experiments.

Solution composition	pH	Rate in light $k_{obs} \times 10 \text{ (hr}^{-1}\text{)}$	Rate in dark $k_{obs} \times 10^2 \text{ (hr}^{-1}\text{)}$
Water	7.28	4.25 ± 0.508	
Water with ascorbate (25 mM) and glutathione (5 mM)	7.25	4.14^a	
Lettuce extract	7.27	5.57 ± 1.08	5.06 ± 6.91
Heat-inactivated lettuce extract	7.25	6.61 ± 0.770	NR ^b
Lettuce extract with ascorbate (25 mM)	7.27	25.1 ± 9.10	10.3 ± 4.60
Heat-inactivated lettuce extract with ascorbate (25 mM)	7.24	8.50 ± 2.66	19.2 ± 2.54
Lettuce extract with glutathione (5 mM)	7.26	4.89 ± 0.907	4.39 ± 4.34
Heat-inactivated lettuce extract with glutathione (5 mM)	7.25	4.40 ± 0.973	5.96 ± 2.45
Lettuce extract with ascorbate (25 mM) and glutathione (5 mM)	7.24	23.4 ± 4.76	49.6 ± 5.50
Heat-inactivated lettuce extract with ascorbate (25 mM) and glutathione (5 mM)	7.24	8.41 ± 0.142	2.89 ± 2.96

^a Predicted value from multivariate model.

^b No reaction.

3.4 Fate of RDX in selected plants

Pigmented and non-pigmented mint tissues, as well as tissues from variegated coleus plants, were examined to determine if the movement of the

RDX to a leaf tissue that exposes the RDX to light at much higher levels than would be present in the soil or in pigmented tissue can lead to photodegradation of RDX. Rather than looking at the transmission spectra of the leaves, we exposed the plants to various wavelengths of light. It is well documented that RDX degrades when exposed to UV and shorter wavelengths of light; it is more stable when exposed to the shorter, red wavelengths. Because plants can grow and thrive under red light, we exposed the green/white coleus plants to RDX while growing under red light. We then compared the RDX accumulation patterns to those seen under white light to determine if the differences seen are attributable to photodegradation or some combination of phyto-photodegradation pathways.

3.5 Photolysis modeling as a probe for biodegradation

The quantum yield for RDX photolysis in aqueous solution was measured over the wavelength range 300–400 nm in 20-nm increments. This allowed the establishment of a kinetic model for RDX loss based on light intensity and leaf thickness, integrated over the wavelengths of interest:

$$-\frac{d[RDX]}{dt} = \phi I (1 - e^{-\epsilon bc}) \quad (3)$$

where

- ϕ = quantum yield for photodegradation
- I = intensity of incident sunlight
- ϵ = molar extinction coefficient for RDX at each wavelength
- b = leaf thickness
- c = concentration of RDX at a given time.

The calculation was integrated with respect to wavelength to deliver the most accurate model possible. The fraction of incident light absorbed by RDX at a given wavelength is given by:

$$\% I_{\lambda}^{RDX} = \frac{\epsilon_{\lambda}[RDX]}{\epsilon_{\lambda}[RDX] + \sum \epsilon_{\lambda}[pigments]} \quad (4)$$

where *pigments* are given by total chlorophyll and carotenoids. Chlorophyll was determined in-situ where possible (Cerovic et al. 2002) and verified by extraction and ex-situ analysis; carotenoids were measured by extraction and ex-situ analysis (Norman et al. 1990). Results from these

models were used to explore the mechanism of RDX loss; accurate prediction by the model would indicate direct photolysis in the aqueous component of the leaf, whereas under-prediction by the model indicates photolysis + enzymatic degradation.

3.5.1 Uptake and accumulation

We have shown differences in leaf accumulation among three plants studied: coleus, peppermint, and tobacco (Table 7). The coleus appeared to have the lowest accumulation of RDX in the leaf tissue, and this appears to be because RDX concentration within the white tissue is lower than in the green, pigmented tissues.

We have shown that preliminary differences in RDX concentrations depend on the color of the leaf tissue when looking at coleus plants with tri-colored leaves (Table 8). We are repeating this experiment for verification as the original sample size was relatively small.

Table 7. RDX uptake and accumulation in three plants.

	Water use (mL)	Total RDX taken up (mg)	RDX in leaves (mg)	% RDX taken up in leaves
Tobacco	275	1.54	0.95	61.95
	250	1.40	1.44	102.86
Peppermint	635	3.56	2.83	79.48
	850	4.76	3.53	74.20
	700	3.92	2.17	55.26
Coleus green tissue	245	1.37	0.27	19.6
	315	1.76	0.59	33.7
	135	0.76	0.16	21.4
Coleus white tissue	245	1.37	0.06	4.4
	315	1.76	0.38	21.5
	135	0.76	0.03	4.2

Table 8. Differences in RDX uptake in tricolored leaves of coleus.

	RDX conc. (ppm)	Standard deviation
Green tissue	1.22	0.52
White tissue	0.75	0.08
Red tissue	0.29	0.09

To investigate the possibility of preferential accumulation of RDX in different areas of the leaf tissue, we have grown plants that have different pigmentation patterns as well as solid green coleus, and have exposed them to RDX. The pigmented leaves were sectioned so we can extract the different pigmented areas separately. The solid green coleus was also exposed to RDX, and the leaves, although solid green, were sectioned in the same manner as the differentially pigmented leaves.

3.5.2 Photodegradation studies

We have performed a multivariate photolysis of RDX in the presence of glutathione, glutamine, cysteine, sucrose, and ascorbate. The effort involved observing RDX photodegradation over 12-hours, sampling at 3-hour intervals, and incorporating approximately 320 photolyses measurements. In every sample, RDX photodegraded; however, it did not correlate to the concentration of these molecules (Fig. 20). From this, we have concluded that RDX will photodegrade given exposure to 300–400 nm photons but that degradation is a wholly intra-molecular process and is probably not related to the molecular content of the cytosol.

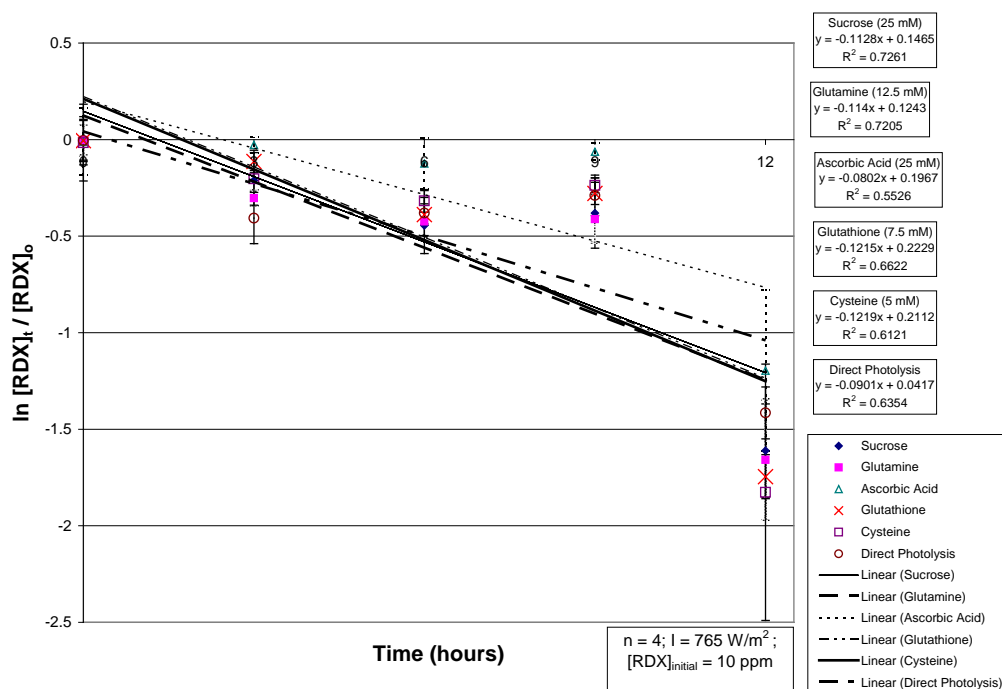


Figure 20. RDX photolysis does not correlate with the presence of several different cytosol components.

We ran corresponding matrices for a series of nitroaromatics (initially nitrobenzene; 3-nitroanisole; 4-nitroanisole; 3-nitrotoluene; 4-nitrotoluene; 3-nitrophenol; 4-nitrophenol; 1-chloro-3-nitrobenzene, and 1-chloro-4-nitrobenzene). Nitrobenzene photolysis appears to be quite sensitive to cytosol components, with glutathione taking the lead in promoting photodegradation (Fig. 21). This strategy of screening cytosol components for their impact on intermolecular photodegradation helps us identify a photoremediative approach where we can select the optimal plant to promote abiotic photodegradation. None of these components have an effect on RDX or NB in the “dark” over a period of weeks. These experiments were not optimized to probe humification processes.

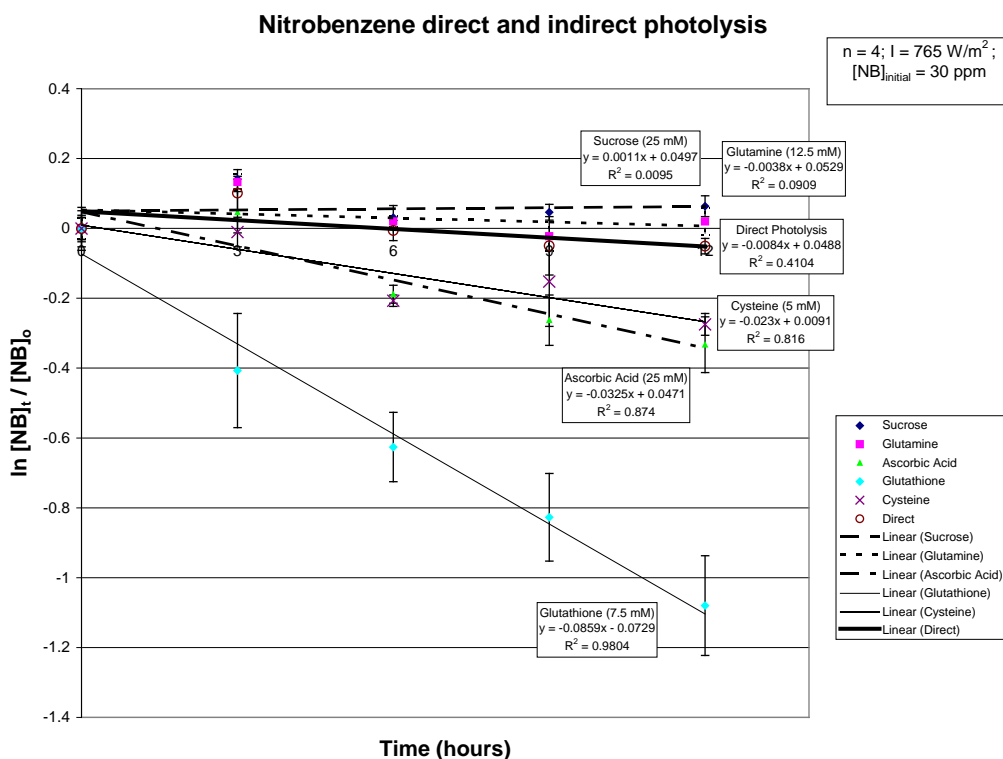


Figure 21. Photodegradation of nitrobenzene correlates strongly with the presence of glutathione. Ascorbate and cysteine may also be significant.

We performed the corresponding dark controls for RDX stability in contact with solutions of the same. In the absence of UV radiation, RDX was stable for the duration of the experiment (2 weeks). The implication is that RDX degradation in the plant is a function of incident solar radiation and that these commonly occurring reductants do not react with it at all in the absence of enzymes. The implications for humification are not known at this time.

4 Task 3: Humification

Soil science uses the term humus to describe organic matter in soil that has reached an essentially constant state. Humification is the process of creating humus. Soil organic matter and the humic material in soils affect many soil biochemical properties.

Mineralization–Immobilization–Turnover (MIT) of Soil Organic Matter (SOM) is a key component of carbon cycling in terrestrial environments. SOM is a pool of carbon from a wide variety of sources, including contaminant and plant-derived C and CO₂. Soils containing high SOM levels located in temperate regions, such as the continental US, are typically vegetated with grasses. Grasses fix a significant amount of CO₂ via photosynthesis, and much of the fixed carbon is ultimately incorporated into SOM by rhizosphere-microbial processes. This pool of carbon can drive MIT processes in a soil towards immobilization–humification. In essence, we are proposing to examine the MIT process in a soil to determine the environmental factors that influence the humification of RDX contamination. In sufficiently reduced systems, RDX has been shown to attenuate, but the specific reactions and characterization of the residues that are produced have not been completely determined (Hawari et al. 2002).

Recent studies have demonstrated that both bacteria and fungi can also mineralize RDX, but, again, the pathways and intermediates formed are poorly understood (Sheremata and Hawari 2000). Because precedence has been established for RDX transformation, and explosives have been shown to covalently bind to soil humic fractions or organic material in compost, a humification approach may have significant utility in treating surface soils on impact and training ranges. On a mass basis, RDX-derived carbon is a minor component of the total carbon in a training or impact range soil and the fate of RDX is almost certainly influenced by the native carbon cycles. Consequently, one approach to remediating RDX in range soils is to manipulate the natural carbon cycle in a manner that favors RDX attenuation. Carbon cycles in soil are greatly influenced by temperature, soil-water potential, and bioavailable carbon. Of these, modifying bioavailable carbon would be the most amenable to a large-scale strategy necessary to treat military ranges. Bioavailable soil carbon can be modified by plants.

Agronomists have shown that high-organic-matter soils reduce the efficacy of the herbicide 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (Atrazine) in surface soils (Benoit and Preston 2000; Sluszny et al. 1999). Structurally, RDX has characteristics of both Atrazine (a triazine ring) and RDX (nitro groups). Therefore, it is feasible that RDX or its breakdown products can become humified. The uptake, transformation, and translocation of RDX in plants have been demonstrated, although complete RDX degradation within plants is less certain (Larson et al. 1999a). A study of the classification of RDX residues in a number of terrestrial plants after RDX uptake from soil showed that the vast majority of the plant-associated labeled carbon was present in large molecular weight biomolecules within the plant tissues (Larson et al. 1999a). A small percentage of the labeled carbon was found to be present as the parent RDX compound. There is a concern by regulators and stakeholders that plants may serve as a pump to cycle RDX and RDX residues from soil to plant, and then back to soil following plant senescence. It is also possible that a soil–plant–soil cycle would reduce the potential for RDX to leach and establish conditions favorable for humification by providing the bioavailable carbon necessary to drive humification processes. The fate of large molecular weight RDX residues following plant senescence has not been well characterized and is an important component of the soil–plant–humic RDX cycle (see Fig. 1).

Although a number of studies have shown that anthropogenic compounds, including explosives, can bind irreversibly to humic substances, many of these studies were conducted in reduced environments (Pennington et al. 1999; Achtnich et al. 1999a, b; Shen et al. 2001). Despite these observations, a thorough understanding of the environmental chemistry and microbiology associated with the reduction and binding phenomena is still lacking (Bruns-Nagel et al. 2000b). Many of these previous studies have used ^{14}C labeled compounds to map the fate of the labeled anthropogenic material. Only a few studies have attempted to associate binding of the anthropogenic to a specific soil fraction (Bruns-Nagel et al. 1999a, b) and even fewer studies have attempted to define the microbial ecology linked to the humification process. By concomitantly quantifying explosive transformations and cycling among various carbon pools, and the dynamics of the associated biochemistry during the humification process, environmental factors that influence the reactions may become apparent. Knowing what these factors are may lead to an understanding of how best to manipulate the in-situ conditions to favor irreversible binding of RDX residues onto surface soils. Understanding these mechanisms may ultimately

lead to the development of a low-cost attenuation strategy for RDX remediation on military ranges.

4.1 RDX transformation

Research has shown that the anaerobic reduction of RDX typically proceeds through the sequential formation of mono-, di-, and tri-nitroso derivatives, followed by the formation of hydrazine (Fig. 22; McCormick et al. 1976) or via direct ring cleavage resulting in the formation of nitroamine and formaldehyde (Hawari et al. 2000). These intermediates can then be further bio-transformed to either nitrous oxide or carbon dioxide. Obtaining a sufficiently low system Eh appears necessary for the reduction process to be initiated. In moderately and well-drained surface soils, gaseous diffusion rates are generally greater than oxygen consumption rates and reduced conditions are atypical. Although microcosm studies can be devised to control Eh and maintain a reduced environment, obtaining long-term reduced conditions on large acreages of surface range soils is as yet unfeasible. However, Eh can be temporarily lowered in surface soils through the combination of high levels of bioavailable carbon, microbial activity, and water content. Although we cannot realistically alter the temperature or water content of large acreages of surface soils on ranges, we can alter the bioavailable carbon in soil through vegetation.

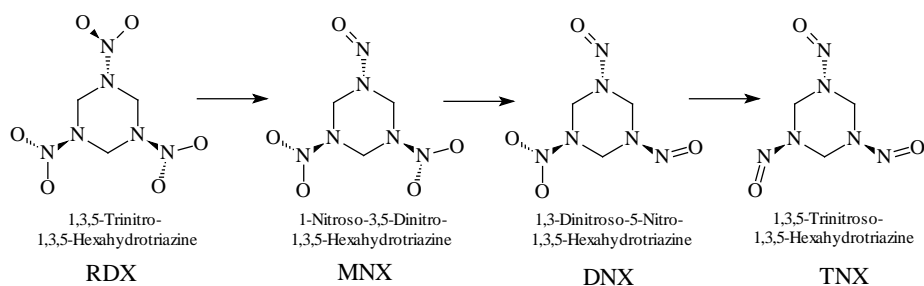


Figure 22. RDX and the reductive transformation products, MNX, DNX and TNX.

Humification, or the incorporation of organic residues into the soil humic fraction, can decrease free or available concentrations of many compounds added to soil. For humification to occur, sufficient bioavailable carbon must be present to support the ongoing formation of humic material. Humification of TNT and TNT residues has been demonstrated in composting and soil systems (Held et al. 1996; Pennington et al. 1999), but humification of RDX is less definitive. Kaplan and Kaplan (1982) were the first to show that the composting of TNT resulted in the formation of azoxy and mono- and diamino intermediates. Pennington et al (1999) have

shown that TNT reduction is followed by covalent bonding of the intermediates, presumably into humic oligomers. Observations of decreasing bioavailability and extractable TNT as compost “ages” suggest that humification is an ongoing process that begins and continues with the microbial degradation of the explosive and energetic contaminants (Palmer et al. 1997).

Yet, the microorganisms involved and how their biochemistry may affect the outcome are less clear. To date, the known factors that impart a major influence on the composting process are limited to compost size, structure, aeration, pH, moisture, and C/N ratio of the substrate (Ro et al. 1998). Drier conditions in soils, as may be found in surface soils, often favor fungal communities over bacterial communities. Fungal communities are major contributors to soil organic matter because of their prolific production of phenolic compounds (Martin et al. 1972).

In addition to composting systems, there are soil-based precedents in which humification has been shown to reduce the bioavailability of explosives in surface soils. In 1997, Hundal et al. reported that after 168 days of incubation, 93% of added ^{14}C -TNT became sorbed to humic materials and that 32–40% of the TNT residue was not recoverable via sequential acidic extractions. They concluded that TNT had become irreversibly bound to the soil. In an anaerobic slurry treatment of TNT contaminated soil, Hiltrud et al. (1998) showed that the complete reduction of TNT also led to an irreversible binding of the reduced products. Moreover, they showed that there was no residual toxicity in the soils. Duan et al. (1998) then showed that the rate of TNT reduction could be influenced by the level of electron donor, such as glucose, that was present. Bruns-Nagel et al. (2000a, b) confirmed the humification process by using ^{15}N NMR techniques to show that TNT and TNT residues were incorporated into specific fractions of the humic material of a soil. Thus, precedence has been established for promoting the irreversible binding of TNT residues into specific humic fractions of a soil.

An issue in the humification of explosives and other xenobiotics is the potential for release of the sequestered compounds. Sequestration, as opposed to covalent binding, is a temporary holding of a compound in an unreactive state. Thus, sequestered contaminants can pose a future health risk. Achtnich et al. (1999a, b) and Bruns-Nagel et al. (2000a, b) have shown that it is possible to monitor the covalent binding of a partially reduced nitroaromatics via immunoassay and ^{15}N NMR spectroscopy. Achtnich et al. (2000) also showed that an initial complete reduction of

TNT enhanced the degree to which the explosives residue became irreversibly or covalently bound. They showed that 2,4-DANT became irreversibly sorbed to the organic matter, but 4-ADNT did not react to the same extent under the same conditions, becoming physically sequestered as opposed to covalently bound. Achtnich et al. (2000) went further to show that the covalently bound, partially reduced nitroaromatics could be further reduced while in the bound state, enhancing the covalent, irreversible binding of the contaminant to the soil and minimizing the potential for future health risks. Adapting these technologies should now make it possible to monitor the humification of cyclic nitramines and estimate or verify a reduction in the potential for future health risks.

4.2 Approach

As noted in earlier, this project brought together three areas of RDX removal and degradation—plant uptake, photodegradation, and humification—and built on preliminary work done by members of this research team and many others in this field. Our aim was to better understand the biochemical and physiochemical transformations of RDX within the plant matrix, as well as how the parent compound and metabolites interact with components of the soil matrix. We used RDX-laden plant tissue to follow RDX humification and fate in soils held at moisture and temperature conditions representative of surface soils on training ranges. We also used variegated plants to characterize phyto- and photo-degradation in pigmented and non-pigmented tissues.

Tissue from plants with high uptake rates were incorporated into ongoing humification studies to track and identify fate of ^{14}C -RDX added either directly to soil or from plant tissue. The underlying hypotheses that task 3 addressed are:

- RDX in plant tissue, which is predominantly in conjugated forms because of plant-based processes, can be further irreversibly tied to soil organic matter following plant leaf senescence.
- Incorporation by humification of the RDX residues into soil organic matter will be related positively to the general turnover of soil organic fractions, a process termed MIT.
- Consequently, soils with high MIT will be better at humifying RDX residues than are soils with low MIT.

To test these hypotheses, we began with three sub-tasks and later added a fourth subtask.

- Characterize the relationships among RDX transformation, soil-water potential, and bioavailable carbon at temperatures representative of surface field soils.
- Determine if humification of RDX-derived carbon occurs and is related to mineralization–immobilization turnover of natural soil carbon, a readily measured process that can be influenced with large-scale practices.
- Investigate the fate of plant-associated RDX as it decomposes in both reducing and humifying conditions representative of those in surface soils.
- Investigate the toxicity of humic material leachate following RDX incorporation, relative to the same without RDX incorporation.

4.2.1 Materials and methods

A schematic of the approach we used is shown in Figure 23. In brief, two soils, with different organic matter contents, were incubated with ^{14}C -labelled RDX applied directly to the soil, and, in matched treatments, with ^{14}C -labelled RDX that had been incorporated into plant tissue in an earlier study. For the latter, the plant-tissue-incorporated ^{14}C obtained from a previous experiment was added directly to the soil. The soils were incubated at constant temperature and moisture conditions for periods of approximately 5 months. Evolved CO_2 and ^{14}C -labelled CO_2 were monitored by pre- and post-scrubbing the headspace air for CO_2 . Periodically, soil cores were taken and analyzed for RDX. Analysis fractions included acetonitrile-extractable RDX in the soil, ^{14}C in the microbial biomass fractions, and ^{14}C in the soil organic matter, which was fractionated further into fulvic, humic, bound humic, and bound lipid fractions.

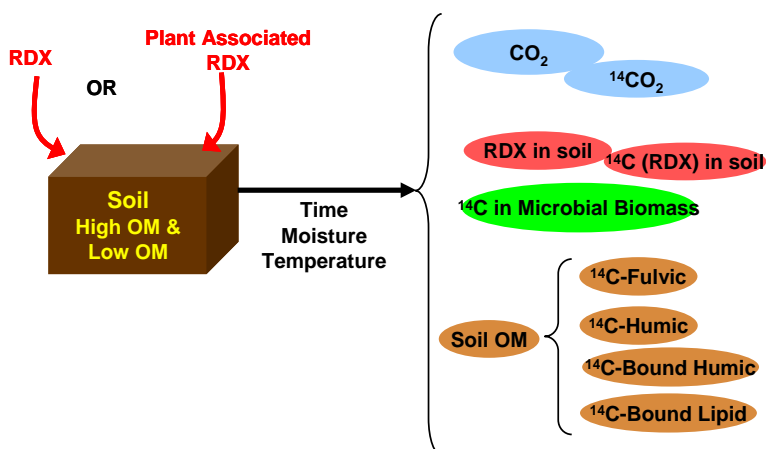


Figure 23. Approach to track and identify fate of ¹⁴C-RDX added either directly to soil or from plant tissue.

4.2.2 Results and accomplishments

For the fulvic and humic soil organic matter characterizations, we used the methyl isobutyl ketone (MIBK) fractionation method described by Nieman et al. (1998) based on earlier work by Rice and McCarthy (1989). In this method, alkaline extraction removes humic (HA) and fulvic (FA) acids from soil based on their solubility, but maintains the structure of added organic compounds. Soil is repeatedly shaken with 0.1 M NaOH to extract FA and HA fractions. This is then repeated until the color of the solution disappears. The intractable fractions are defined as Humin-mineral component, and the supernatant is defined as Fulvic-mineral component. An outline of this method and the color change in the organic fractions are shown in Figures 24 and 25.

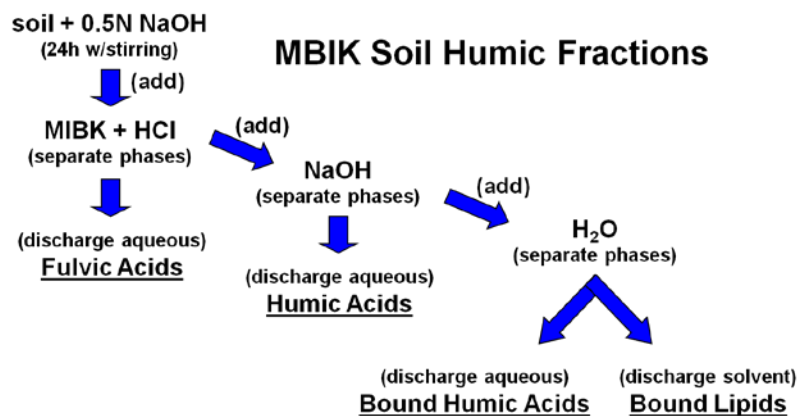


Figure 24. Soil organic matter fractionation scheme.

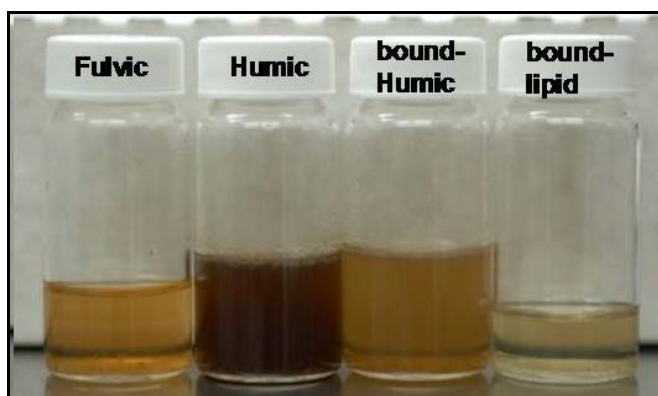


Figure 25. Color changes in the organic fractions following soil extraction.

When ^{14}C -RDX was applied directly to the soil, the amount of ^{14}C in the organic material, expressed as humic:fulvic ratio, was significantly higher in the high organic soil than in the low organic soil. However, when ^{14}C -RDX was applied as plant-tissue associated RDX (+Leaf in Fig. 26), the amount of ^{14}C label that was bound into the humic:fulvic ratio reached similar ratios for both soils by 80 days of incubation. These data support the hypothesis that MIT is driven by bioavailable carbon, and MIT in turn drives humification, or soil organic matter association of ^{14}C derived from RDX.

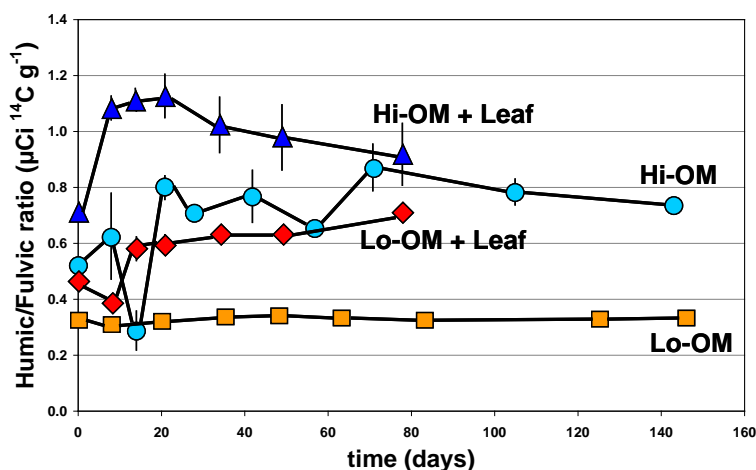


Figure 26. Humic/fulvic ratios in high and low OM soils treated with either ^{14}C -labelled RDX applied directly to the soil or ^{14}C -labelled RDX that was incorporated into plant tissue by growing plants in ^{14}C -labelled RDX in soil (+ Leaf).

Further supporting data are shown below in Figure 27. These data show that microbial biomass is relatively constant and uniform for both the con-

trol (no RDX, no plant tissue) and the RDX additions in the high-organic-matter soil during the incubations, suggesting that the system is in near steady state condition microbially. However, in the low-organic-matter soil, both the control and the RDX treated soils have highly variable microbial biomass, suggesting greater changes in the microbial populations.

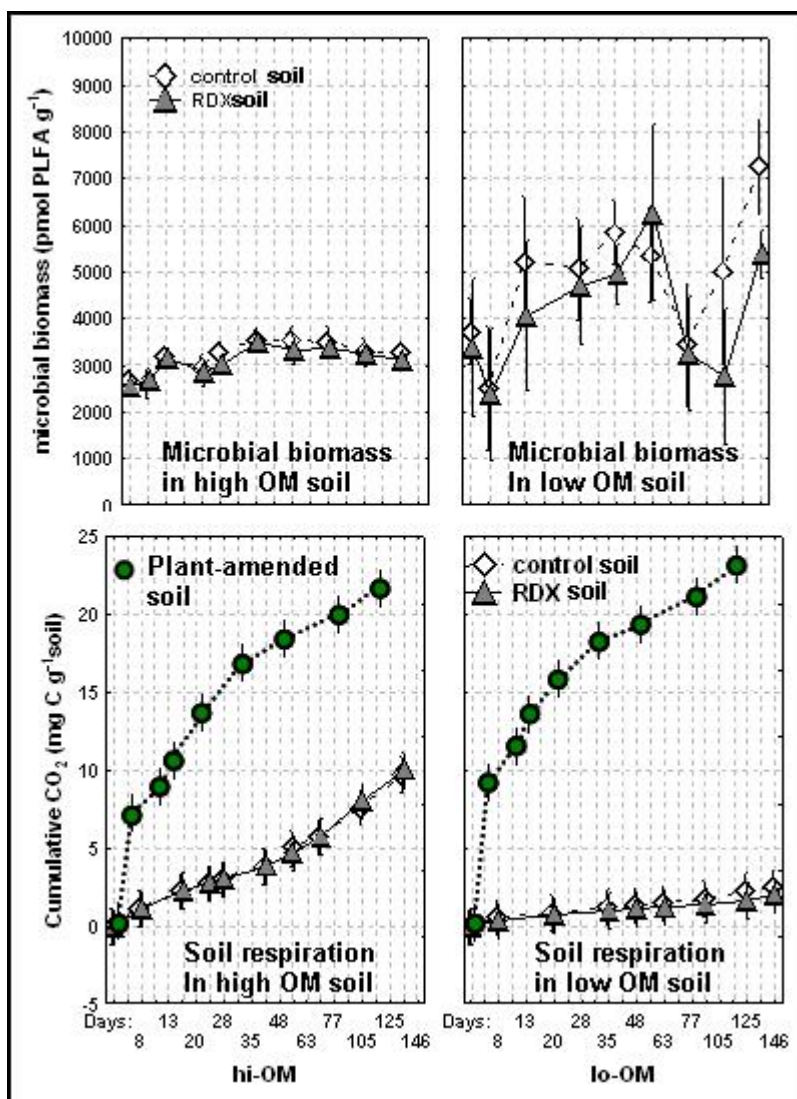


Figure 27. Microbial biomass in high-organic soil and low-organic soil, with and without RDX additions (upper two graphs). Soil respiration expressed as evolved CO₂ in high-organic soil and low-organic soil, with and without RDX additions, and with plant tissue and associated RDX amendment (lower two graphs).

The lower graphs in Figure 27 show the cumulative CO₂ flux from the soils. Without plant tissue additions, the high organic matter soil has greater evolved CO₂ than does the low organic soil. The addition of plant tissue increased evolved CO₂ for both soils, and the CO₂ evolution curves were

nearly identical for both soils. These data show that the addition of readily bioavailable carbon, such as plant tissue, resulted in similar evolved CO_2 for both soils and overcame inherent soil organic matter differences. These data were also in agreement with, and support, greater MIT and greater ^{14}C incorporation in the humic:fulvic fraction of the soils.

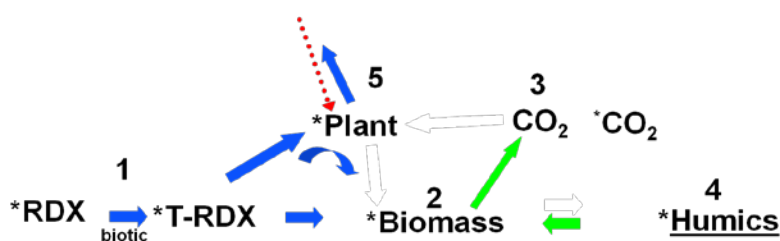


Figure 28. Summary of significant pathways in RDX fate in soil-plant-microbial systems. Numbers associated with each labile pool of RDC-derived C are discussed below.

Figure 28 summarizes the findings of the importance of the steps first shown in Figure 1.

1. Biotic transformation of RDX in surface soils to RDX transformation products (T-RDX) was slow but does occur.
2. Accumulation of ^{14}C in soil biomass (*Biomass) was low but constant—suggesting steady state role in flow of RDX into other pools.
3. Evolution of $^{14}\text{CO}_2$ (* CO_2 ↑) was low but constant. General CO_2 evolution (CO_2 ↑) may be important as an indicator of MIT.
4. For RDX applied directly to the soil, we observed greater amounts of ^{14}C (*C) associated with the bound-humic fraction in the high OM (organic matter), high respiration soil relative to the lower OM soil. For RDX applied from plant-tissue-associated RDX, we observed changes in ^{14}C (*C) in humic fractions for both soils, but more so for low OM soil.
5. Photo-degradation in plant tissue using variegated plants was described in an earlier section, using variegated plants.

The practical implications of these data are that humification of RDX into the soil organic fraction was enhanced by: 1) “preconditioning” the RDX by plant-associated conjugation processes that occur after plant uptake and 2) increasing MIT in the soil. Both of these enhancements can be favorably influenced by exploiting the plant-uptake, conjugation, and plant senescence cycles.

4.2.3 Leachate toxicity

We designed a test for the toxicity of leachate from humic material with and without RDX incorporation or humification. There are limits imposed by the nature of the previous studies in which we generated soil that had RDX and plant-associated RDX bound to or in the humic fraction. Because the mass of soil remaining from the earlier humification tests was limited, we sought a modified toxicity test that could be done with minimal soil and yet had relatively high sensitivity. Additionally, earlier work on the toxicity of leachate from other explosive treatment studies, notably composting, have shown that control, or non-explosive amended compost, had relatively high toxicity that appeared to be, for the tests used, inherent to the compost.

We used an algal-based test that requires small masses of soil, is relatively sensitive, and is based on *Selenastrum capricornutum* growth inhibition.

Owing to the small amounts of soil that remained from the earlier humification incubations, there were constraints on the comparisons that we could make. The comparisons were: soil vs. plant; low OM vs. high OM; pre-incubation vs. post-incubation; and RDX vs. no RDX (Fig. 29).

A synopsis of the methods follows. Available soils were processed via a modified TCLP batch assay. A slurry of 5 g soil in 40 mL H₂O was shaken at 30 rpm for 24 hours. The slurry was then centrifuged at 10,000 × *g* for 10 minutes to pelletize the soil. The supernatant was recovered and preserved at 4°C for the micro-algae toxicity test. An additional 40 mL of H₂O was added to the soil pellet, and the supernatant recovered and stored. The soil pellet was then extracted with 40 mL of 2N acetic acid and the supernatant collected and stored. We observed no significant differences in toxicity of the supernatant from RDX treated soils relative to control soils, as shown in Figure 29.

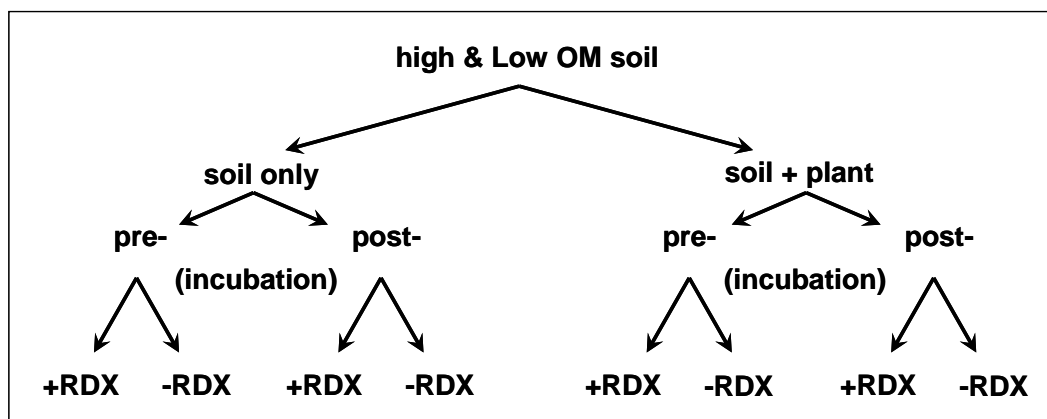


Figure 29. Treatments that were tested using a modified leachate toxicity test based on the growth inhibition of the algae *Selenastrum capricornutum*.

4.3 Conclusions

For soils incubated under non-saturated, aerobic conditions representative of surface range soils, we have observed differences in ^{14}C -RDX fate in high-organic-matter (OM) soils relative to low OM soil. Greater OM was positively related to greater ^{14}C in the bound-humic fraction.

Additionally, our data showed that adding the ^{14}C conjugated in plant material to the high OM soil was, after 80 days, yielding ^{14}C in humic:fulvic ratios that were similar to ratios measured after adding ^{14}C -RDX directly to the soil.

For the low OM soil, ^{14}C in the humic:fulvic ratio after adding plant-associated ^{14}C -RDX more closely followed the high OM soil, and similar amounts of ^{14}C (from RDX) became associated with bound humic fractions.

These data support the concept of humification and suggest that MIT processes, which can be favorably influenced by soil OM management, may have utility as a tool for affecting the fate of RDX residues in favorable ways. Current studies are investigating changes in toxicity of leachate from soil humic material that has RDX moieties incorporated, relative to non-RDX humic material.

5 Conclusions

5.1 Task 1

As demonstrated by the all-green coleus leaf tissue, RDX is translocated preferentially across the leaf surface and accumulates at higher levels in the outer edges. It would be beneficial in future research to test this theory by exposing different uni-colored coleus varieties to RDX and verifying that they have the same dispersion pattern. It would also be beneficial to test this theory with plants from other genera.

Chlorophyll concentration affects RDX concentration; higher chlorophyll content results in elevated RDX concentrations in the leaves. As evidenced by the green-red coleus, this effect of chlorophyll concentration on RDX degradation is more important than simple translocation; when chlorophyll concentration is exceptionally low, then RDX degrades quickly and does not accumulate.

The presence of anthocyanins in the leaf tissue has no effect on RDX concentration. When anthocyanins are present in tissues that would otherwise be similar—green-white coleus compared to tri-color coleus and in the dark red-green coleus compared to all green coleus—there is no change in the ratio of RDX concentration.

When the chocolate mint was compared to the other mint species tested, it exemplified the idea that higher chlorophyll concentration resulted in a higher RDX concentration within the leaf tissue. However, when compared to the green coleus tissues tested, it seems that the opposite was true and it can therefore only be concluded that an accelerated rate of degradation was taking place in the chocolate mint. It would be very beneficial to screen more mint varieties to see what others may show this difference in RDX levels. We speculate that there may be RDX degradation attributable to secondary enzymatic activities within the leaf tissues of the chocolate mint; elucidating these enzymatic pathways and identifying RDX metabolites would be necessary to confirm this theory.

Based on the findings of this research, when selecting plants to use for the phytoremediation of RDX, a good selective marker would be the concentration of chlorophyll in the leaf tissues. In particular, plants with large

unpigmented areas or with relatively low chlorophyll concentrations would be excellent plants to select for field studies or use.

5.2 Task 2

Results of the photolysis studies were consistent with respect to the role of ascorbate in TNT photolysis. In the extract photolysis, the presence of ascorbate (25 mM) in fresh extract increased the rate of photolysis by 4.3-fold compared to fresh extract with no ascorbate (Fig. 19). Ascorbate increased the rate of photolysis by 3.0-fold in fresh extract compared to heat-inactivated extract (Table 6). As there was negligible loss of TNT in the dark control experiments, these results suggest that the presence of ascorbate and sunlight activates an enzyme that degrades TNT.

The photolysis rates were 0.489 and 0.557 hr⁻¹ for the extract experiments with and without glutathione, respectively. Based on rate comparisons, glutathione's effect was negative; however, the magnitude of the effect is negligible considering the calculated error (Table 4).

5.3 Task 3

To date, for soils incubated under non-saturated, aerobic conditions representative of surface range soils, we have observed differences in ¹⁴C-RDX fate in high (OM) soils relative to low OM soil. Greater OM is related positively to greater ¹⁴C in the bound-humic fraction.

Additionally, our data show that adding the ¹⁴C conjugated in plant material to the high OM soil is, after 80 days, yielding ¹⁴C in humic:fulvic ratios that are similar to ratios measured after directly adding ¹⁴C-RDX to soil.

For the low OM soil, ¹⁴C in the humic:fulvic ratio after adding plant-associated ¹⁴C-RDX more closely followed the high OM soil, and similar amounts of ¹⁴C (from RDX) associated with bound humic fractions.

These data support the concept of humification and suggest that MIT processes, which can be favorably influenced by soil OM management, may have utility as a tool for affecting the fate of RDX residues in favorable ways. A biological assay showed toxicity of leachate from soil humic material that had RDX moieties incorporated was not significantly different from the non-RDX humic material.

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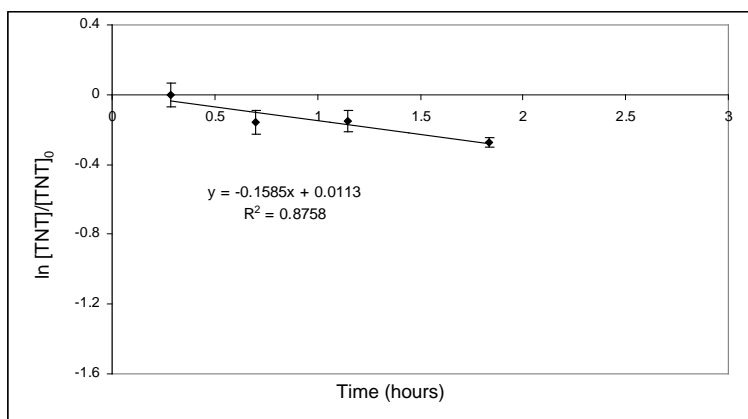
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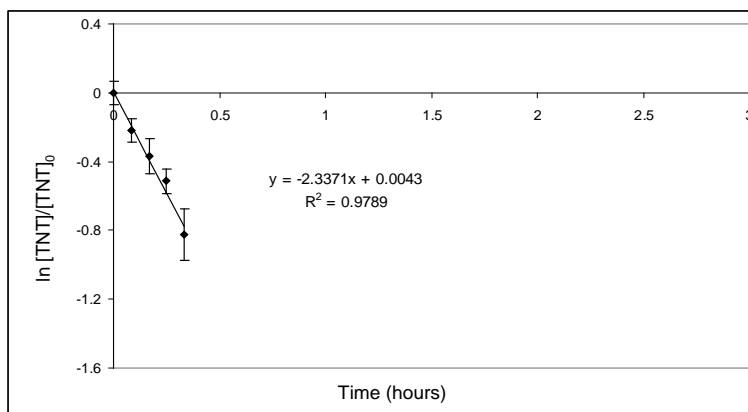
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Appendix A: Supporting Data

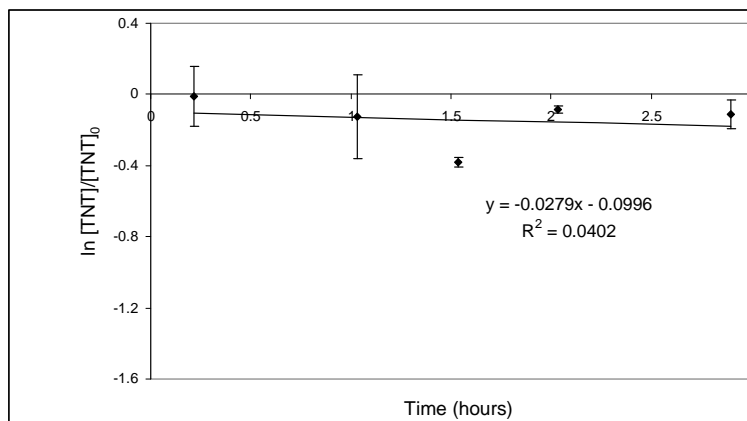


a. First order plot of TNT degradation in dark conditions.

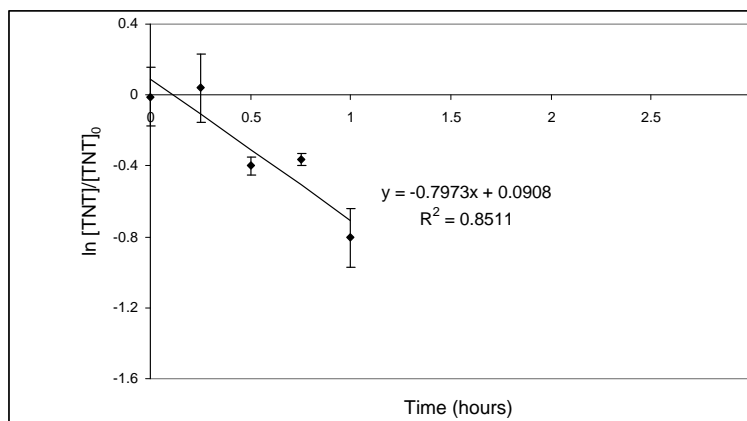


b. First order plot of TNT degradation in light conditions.

Figure A1. TNT degradation in iceberg lettuce extract with ascorbate (25 mM), glutathione (5 mM), and pH = 7.24. The rate of TNT degradation (k_{obs} = slope) is shown for light and dark experiments ($n = 3$).

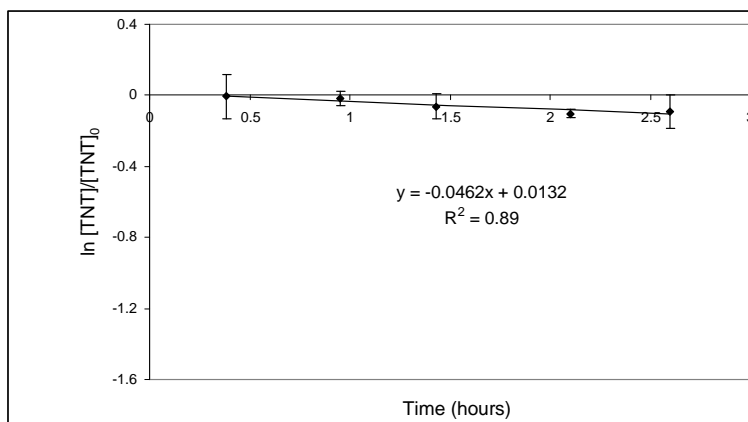


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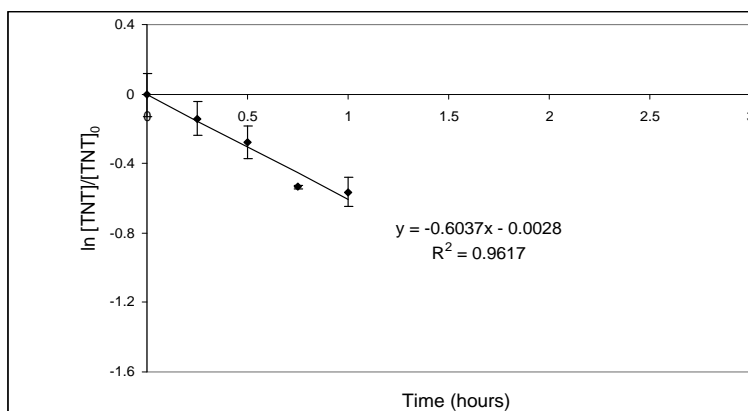


b. First order plot of TNT degradation in light conditions.

Figure A2. TNT degradation in heat-inactivated iceberg lettuce extract with ascorbate (25 mM), glutathione (5 mM), and pH = 7.24. The rate of TNT degradation is shown for light and dark experiments ($n = 3$).

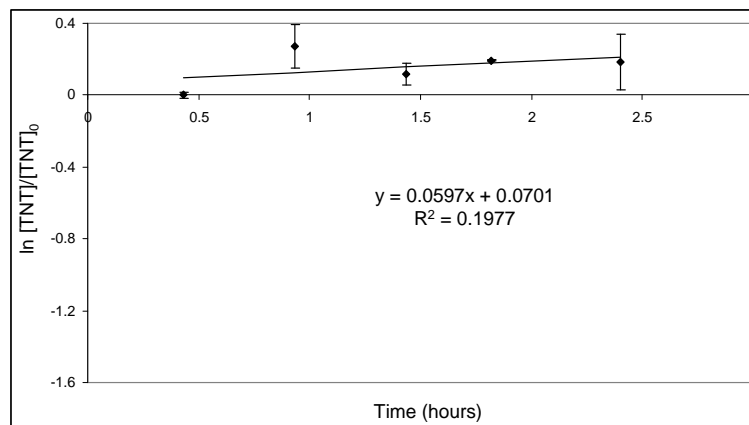


a. First order plot of TNT degradation in dark conditions.

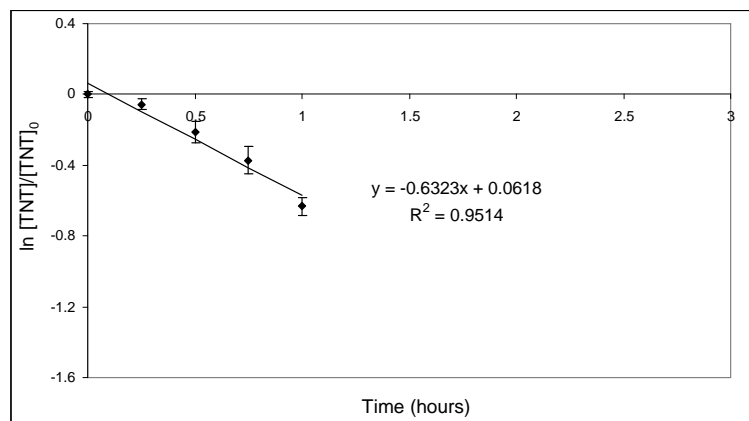


b. First order plot of TNT degradation in light conditions.

Figure A3. TNT degradation in iceberg lettuce extract with pH = 7.27. The rate of TNT degradation is shown for light and dark experiments ($n = 3$).

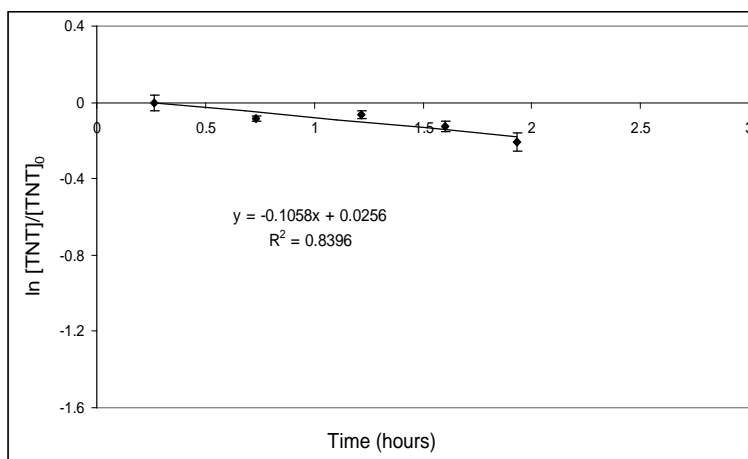


a. First order plot of TNT degradation in dark conditions.

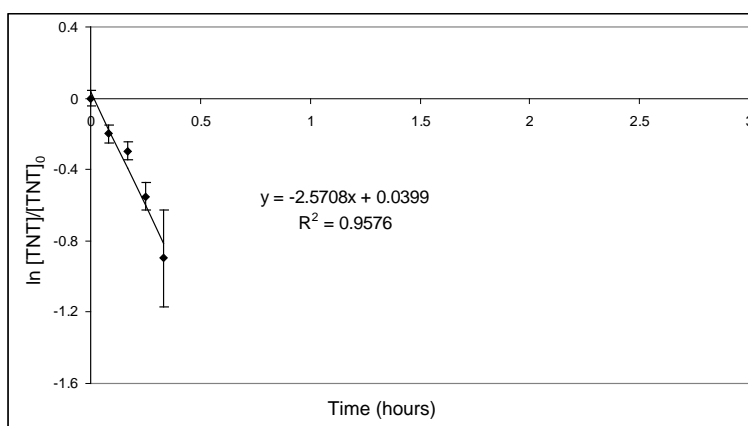


b. First order plot of TNT degradation in light conditions.

Figure A4. TNT degradation in heat-inactivated iceberg lettuce extract with pH = 7.25. The rate of TNT degradation is shown for light and dark experiments ($n = 3$).

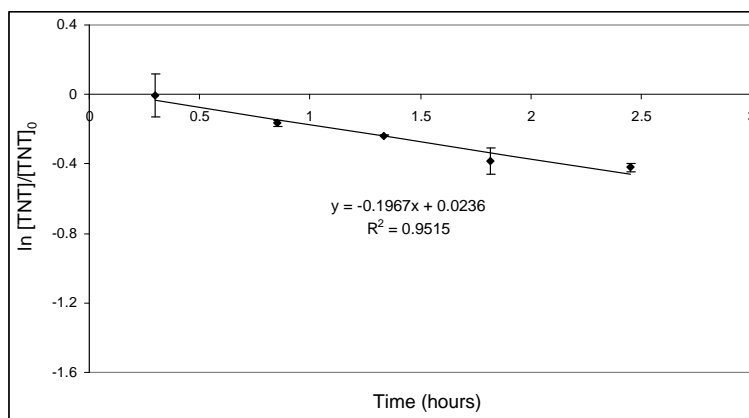


a. First order plot of TNT degradation in dark conditions.

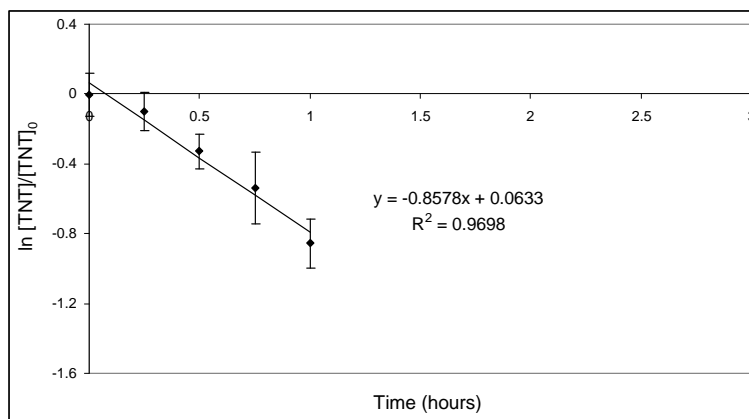


b. First order plot of TNT degradation in light conditions.

Figure A5. TNT degradation in iceberg lettuce extract with ascorbate (25 mM) and pH = 7.25. The rate of TNT degradation is shown for light and dark experiments ($n = 3$).

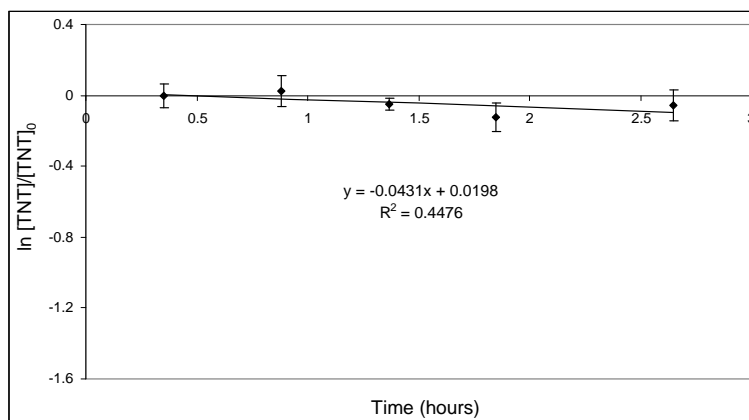


a. First order plot of TNT degradation in dark conditions.

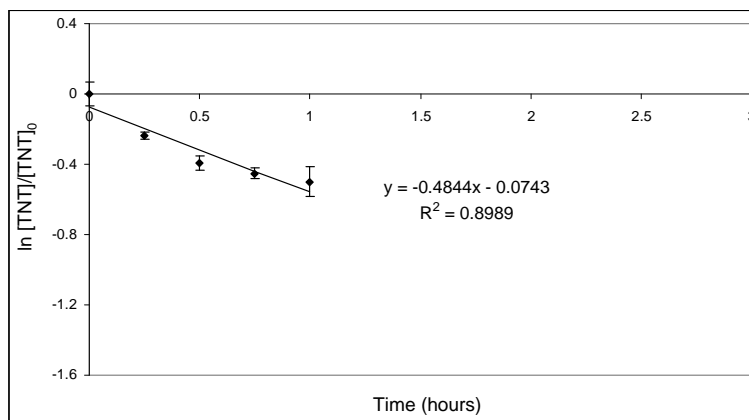


b. First order plot of TNT degradation in light conditions.

Figure A6. TNT degradation in heat-inactivated iceberg lettuce extract with ascorbate (25 mM) and pH = 7.24. Rate of TNT degradation is shown for light and dark experiments ($n = 3$).

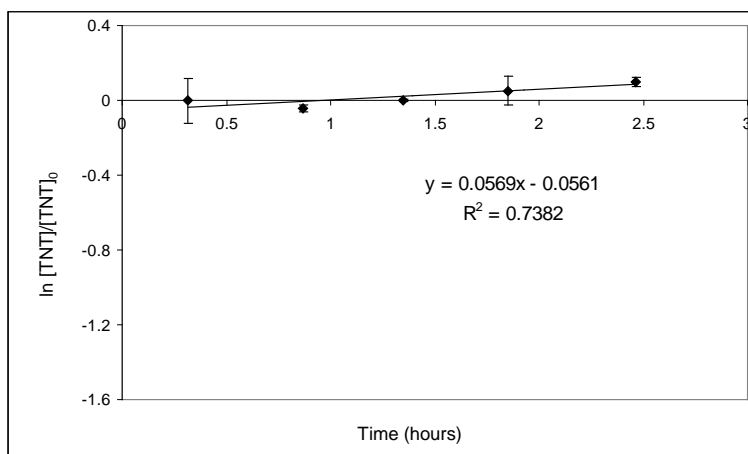


a. First order plot of TNT degradation in dark conditions.

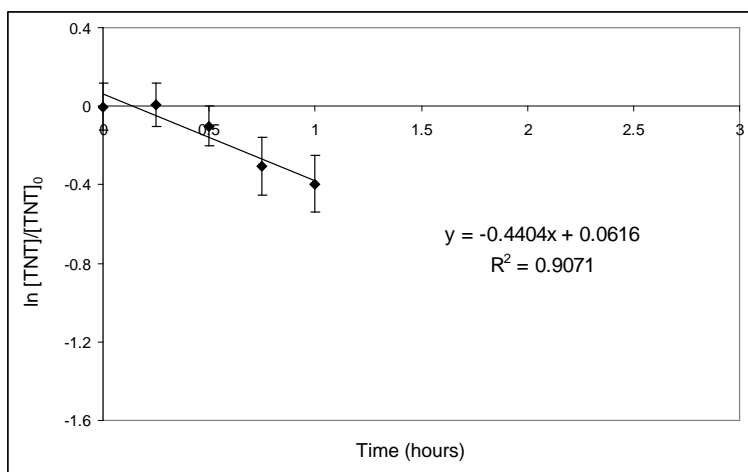


b. First order plot of TNT degradation in light conditions.

Figure A7. TNT degradation in iceberg lettuce extract with glutathione (5 mM) and pH = 7.26. The rate of TNT degradation is shown for light and dark experiments ($n = 3$).



a. First order plot of TNT degradation in dark conditions.



b. First order plot of TNT degradation in light conditions.

Figure A8. TNT degradation in heat-inactivated iceberg lettuce extract with glutathione (5 mM) and pH = 7.25. Rate of TNT degradation is shown for light and dark experiments ($n = 3$).

Appendix B: List of Technical Publications

Technical reports

- Ringelberg, D. B., C. M. Reynolds, L. B. Perry, and K. L. Foley . 2005. Effect of *acetonitrile on RDX biodegradation in an unsaturated surface soil*. ERDC/CRREL TR-05-5.
- Ringelberg, D. B., C. M. Reynolds, K. L. Foley, and L. B. Perry. 2005. *Microbial community shifts associated with RDX loss in a saturated and well-drained surface soil*. ERDC/CRREL TR-05-4.
- Hadden, C.B. B. 2007. *The phyto- and photodegradation of RDX by plants in the Lamiaceae family*. MS Thesis, University of South Carolina
- Rahn, T. W. 2007. *Identification of significant factors in the photophotolysis of 2,4,6-trinitrotoluene*. Master of Science Thesis, Department of Chemistry and Biochemistry, College of Arts and Sciences, University of South Carolina

Published technical abstracts

- Reynolds C. M., D. B. Ringelberg, and S. L. Larson. 2007. Humification of plant-conjugated energetics following leaf drop: Carbon fate and humic fraction toxicity. In *Program Guide, Partners in Environmental Technology Technical Symposium and Workshop, Washington DC, 4–7 December 2007*, SERDP-ESTCP, Arlington, VA.
- Reynolds C. M., D. B. Ringelberg, S. L. Larson, and N. R. Adrian. 2005. RDX humification potential in surface soils and mineralization immobilization turnover. *8th International in Situ and On-site Bioremediation Symposium 2005 (Battelle Conference) 5–9 June*.
- Reynolds, C. M., D. B. Ringelberg, L. Newman and S. L. Larson. 2005. Humification of plant-tissue associated RDX. In *Program Guide, Partners in Environmental Technology Technical Symposium and Workshop, Washington DC, 29 November –1 December 2005*, SERDP-ESTCP, Arlington, VA., p F-27.
- Reynolds, C. M., D. B. Ringelberg, L. Newman and S. L. Larson. 2005. Phytoremediation—Humification strategies for RDX in surface Soil. *Third International Phytotechnologies Conference, Atlanta, GA, 19–22 April 2005*.
- Reynolds, C. M., L. A. Newman, D. B. Ringelberg, and S. L. Larson. 2005. Invited Presentation—*Plant-Soil-Microbial Cycles Leading to Live-Fire Range Sustainability, Phytoremediation Session—21st Annual International conference on Soils, Sediments, and Water, 17–20 October 2005, Amherst, MA*.
- Reynolds, C. M, D. B. Ringelberg, S. L. Larson, and N. R. Adrian. 2005. RDX humification potential in surface soils and mineralization immobilization turnover. In *2nd Sustainable Range Management Conference and Exhibition, 22–25 August, San Antonio, TX*.

Reynolds, C. M., D. B. Ringelberg, S. L. Larson, and N. R. Adrian. 2004. RDX fate in surface range soils—Potential for humification. *1st Sustainable Range Management Conference and Exhibition, January, New Orleans, LA.*

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1. REPORT DATE (DD-MM-YYYY) January 2013		2. REPORT TYPE Technical Report		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE RDX in Plant Tissue: Leading to Humification in Surface Soils				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Charles M. Reynolds, David B. Ringelberg, Karen L. Foley, Lee A. Newman, Carrie B.B. Hadden, Trey W. Rahn, and John L. Ferry				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cold regions Research and Engineering Laboratory US Army Engineer Research and Development Center 72 Lyme Road Hanover, NH 03755				8. PERFORMING ORGANIZATION REPORT NUMBER ERDC/CRREL TR-13-4	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Strategic Environmental Research and Development Program Arlington, VA				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The overall objective was to improve the understanding of RDX transformation in plant tissues and the subsequent cycling of tissue-associated RDX and RDX daughter products among soil mineral and humic fractions following plant senescence. The hypothesis was that environmental risks from RDX at military training ranges can be reduced, and possibly eliminated, through a series of coupled processes involving plant uptake, plant enzyme mediated transformation, photodegradation in the plant, and finally humification of plant-tissue-associated RDX conjugates into soil organic matter after plant senescence and leaf drop. Although the effect of each individual process may be small, the combined effects of the processes taken as a system for sustainability may have a significant impact on RDX residues on surface soils. If so, they may lead to feasible range sustainability management practices. RDX is found in the soils and groundwater of bombing ranges and manufacturing sites. Plants of the family Lamiaceae were used to determine if either their enzymatic activities could accelerate the degradation of RDX once taken up from an aqueous solution. Plant tissue with higher chlorophyll content was found to contain higher concentrations of RDX, while the presence of anthocyanin appeared to have no impact. Of the four varieties of mint tested, chocolate mint, a variety of spearmint [<i>Mentha spicata</i>], had significantly lower RDX concentrations in its leaf tissues. Further research is needed to determine what processes are responsible for the reduced RDX content.					
15. SUBJECT TERMS Enzyme mediated transformation Humification		Military training ranges Photodegradation Plant uptake		RDX pollution RDX transformation TNT pollution	
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
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14. ABSTRACT (Cont'd).

Ascorbate, pH, and glutathione (GSH) were found to be statistically significant factors in the photodegradation of 2,4,6-trinitrotoluene (TNT), a process applicable to RDX. Ascorbate and pH increased the rate of TNT degradation, whereas GSH inhibited it. Photo-induced degradation of TNT occurs at approximately the same rate in extract-based solution. The results indicate that ascorbate and pH increase the rate of photolysis of TNT, whereas glutathione decreases it. In sufficiently reduced systems, RDX has been shown to attenuate, but the specific reactions and characterization of the residues that are produced have not been completely determined. Recent studies have demonstrated that both bacteria and fungi can also mineralize RDX, but, again, the pathways and intermediates formed are poorly understood. Because precedence has been established for RDX transformation, and explosives have been shown to bind covalently to soil humic fractions or organic material in compost, a humification approach may have significant utility in treating surface soils on impact and training ranges.